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Edited by
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THE AMERICAN
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VOL. 2 JANUARY, 1922 No. 1

INVESTIGATIONS ON THE CONTROL OF HOOKWORM DISEASE.

II. The Description of an Apparatus for Isolating Infective Hookworm Larvae from Soil.*

By WILLIAM W. CORT, Ph.D., JAMES E. ACKERT, Ph.D., DONALD L. AUGUSTINE, B.S., and FLORENCE KING PAYNE, M.D.

(Received for publication, September 20, 1921.)

Introduction.

Little information is available on the life of mature hookworm larvae in soil on account of the difficulty experienced in studying them in their natural environment. Not until the investigations of Baermann (1917) was an apparatus devised with which hookworm larvae can be isolated from considerable quantities of soil. This apparatus makes it possible to study the activities of the infective hookworm larvae in the soil and to determine accurately the sources of infestation with hookworm disease in any region. A modification of Baermann's apparatus has been used with success in the investigations of the past summer. On account of the importance of this apparatus and because Baermann's description is published in a rather inaccessible place we will describe in detail the apparatus which we used for the isolation of hookworm larvae from soil and the experiments which were performed to standardize it under different conditions of soil, moisture and temperature.

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* This paper is the first report from the investigation in Trinidad, British West Indies, on the life of hookworm eggs and larvae in soil, carried on by the Department of Medical Zoology of the School of Hygiene and Public Health of the Johns Hopkins University, with the cooperation of the International Health Board of the Rockefeller Foundation. The expedition worked in connection with the intensive hookworm campaign in Trinidad of which Dr. George C. Payne of the International Health Board was director, and was under the direction of Dr. William W. Cort of the Johns Hopkins University. The expenses of the expedition were paid by the International Health Board. The location of the work in Trinidad was made possible by the interest and cooperation of the Government of Trinidad and Tobago, who through the Surgeon-General, Dr. K. S. Wise, and other officers tendered to the expedition many courtesies which aided in the successful conduct of the work. Those who took part in the investigations were Dr. James E. Ackert, of the Kansas State Agricultural College; Mr. D. L. Augustine, of the Johns Hopkins University; Dr. Florence King Payne, Dr. George C. Payne and Dr. William W. Cort.

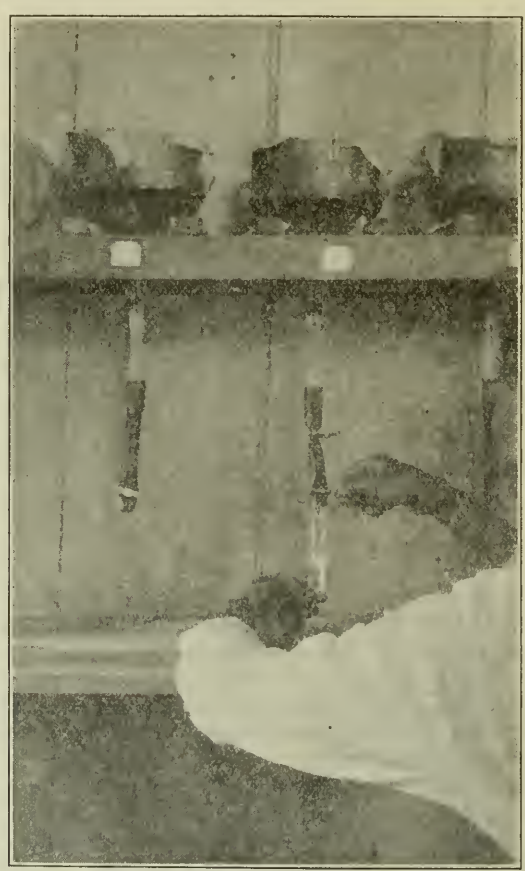

FIG. 1. Two units of the isolating apparatus, showing method of drawing off the water sample.

DESCRIPTION OF ISOLATING APPARATUS.

The apparatus for the isolation of hookworm larvae from the soil works by bringing the lower surface of a soil sample into contact with water of a considerably higher temperature. Under these conditions a large proportion of the nematodes in the sample will pass into the water and can be collected and counted. This isolating apparatus consists of a glass funnel almost filled with water, which has the outlet closed by a clamped piece of rubber tubing. The soil sample is placed in a sieve, which is then fitted down into the funnel so that the level of the water is above the lower surface of the soil. For the examination of soil samples of half a pint or more there were used large glass funnels eight inches in diameter and specially prepared brass sieves seven inches in diameter and three inches in height, with a 1 mm. mesh. To prevent small particles of soil from sifting through into the funnels the sieves were lined with one or two thicknesses of cloth. Figures 1 and 2 show the isolating apparatus set up for use. It will be noted that tightly fitting rubber tubes closed with Hoffman clamps are placed on the stems of the funnels. Nineteen units of this apparatus were in constant use during our investigations. For convenience, as shown in the figures, supports of suitable height were made, and the funnels held in place by padded cross bars. It was found early in the work that if the cross bars were so far apart that the funnels were supported by their rims, considerable breakage re-

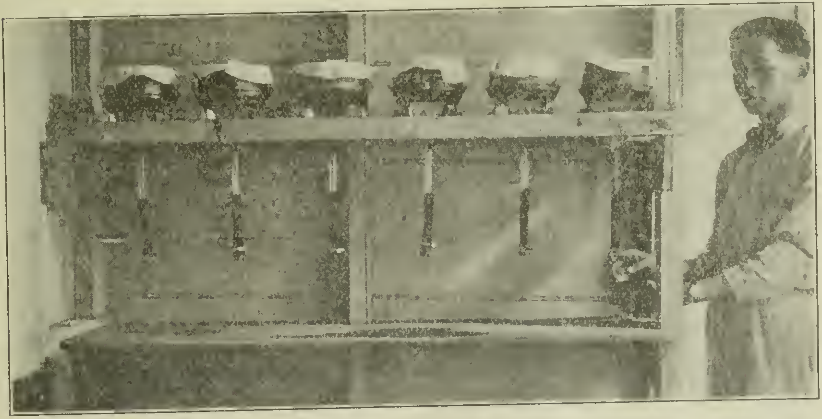

FIG. 2. Six units of the isolating apparatus, showing method of support.

sulted. It is possible to substitute for the sieve a piece of wire screen covered with cloth, of such a size that it can be fitted down into the funnel. The sieves have the advantage of being more easily handled in the transfer of soil.

TECHNIC OF USING THE ISOLATING APPARATUS.

In our routine procedure the soil was thoroughly broken up before being emptied into the sieves. Precautions should be taken not to carry over a little of the soil from one sample to another, and to keep the bottom of the sieve from contact with any dirt. The water used in the funnels was usually heated to a temperature of about 115° F. A funnel, after being set up, was filled within an inch and a half of the rim, so that when the sieve was placed in position there would be no contact between the water and the bottom of the sieve. Water of the same temperature was then poured over the rim of the funnel, until it was practically full and the level of the water almost an inch above the bottom of the sieve. The samples were usually set up in the isolating apparatus some time during the day and left standing over night before the examinations were made. One method of drawing off the samples of water for microscopical examination was to attach a second clamp higher on the tube and to allow the water between the two clamps to run off into a 50 c.c. centrifuge tube (see Fig. 1). A simpler method is to slightly open the clamp at the bottom of the rubber tube and let the water run out until the centrifuge tube is filled. After centrifugation the supernatant water was drawn off with a long pipette and the residue spread out on a two-by-three-inch slide, for microscopical examination. In some instances, when the soil sample was of saturated clay or very finely divided sand, a considerable amount of sediment would fall into the funnel. This could, if necessary, be reduced in amount by running it through a smaller isolating appartus before microscopical examination.

For the examination of small soil samples it was found to be convenient to use a funnel about 9 cm. in diameter. Since no small sieves were available, pieces of screen, bent into a cone shape and lined with cloth, were used for holding the soil samples. The smaller apparatus effected a considerable saving of time in the examinations of small samples, and the standardization tests with it (see Table IV) were somewhat better than those with the large apparatus.

Identification of mature hookworm larvae.

In samples taken in the field, or from experiments where the soil had not been previously heated, there were almost always present nematodes other than hookworm larvae. Baermann (1917 *a*, p. 629) used about one to five drops of a 7.5 per cent. solution of caustic soda to kill these nematodes in the microscopical preparations. We found that a few drops of 5 per cent. formalin, mixed with the water on the slide, would kill the unsheathed nematodes, while the mature hookworm larvae, both sheathed and unsheathed, would remain active for some time. It was not enough, however, after such treatment merely to count the actively moving worms, since the unsheathed hookworm larvae soon quieted down, and sheathed larvae of other kinds were often present which remained active. Since mature unsheathed hookworm larvae were found in large numbers in soil, both under natural and experimental conditions (see Cort, et al., 1922), it was necessary to learn to identify accurately both the sheathed and unsheathed forms. The mature hookworm larvae, both before and after losing their sheath, can be definitely distinguished from all the other kinds of nematodes which have been present in the soil samples.* They show considerable variation in size, but their structure and movements are characteristic. The points of structure which were found most helpful in identifying mature hookworm larvae were (1) the shape and relative length of the esophagus, (2) the general shape of the body, (3) the position of the anus and shape of the tail, (4) the characteristic buccal cavity, (5) the small size of the reproductive primordium, and (6) the peculiar refractivity of the cells of the intestinal wall which gives these worms a color and sheen different from any of the other nematodes found. Besides, the movements of both the sheathed and unsheathed hookworm larvae are very characteristic. We found it helpful at first to keep constantly at hand preparations of hookworm larvae from cultures, to compare with doubtful forms from the soil samples. Later, however, we became so well acquainted with hookworm larvae that there was no further difficulty in making the distinction. The difficulty which certain observers seem to have encountered in distinguishing hookworm larvae from other nematodes can easily be obviated by a sufficient study of their structure and movements, before taking up work requiring indentification.

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* Since *Ancylostoma duodenale* is very rare in Trinidad we can assume that the great majority of the hookworm larvae found belonged to *Necator americanus*.

METHODS OF CULTURING HUMAN FECES TO SECURE HOOKWORM LARVAE.

By the technic described above almost a thousand soil samples, both from the field and from experiments, were examined for mature hookworm larvae during the investigations of our expedition in Trinidad. In order to determine the accuracy of the isolating apparatus with different types of soil and to discover the conditions under which it worked best, a number of experiments were performed on soil samples containing known numbers of mature hookworm larvae. For these and other experiments it was necessary to have available large numbers of mature hookworm larvae. The method by which such large numbers of these larvae were obtained in condition suitable for use may be of help to others undertaking similar investigations so that they will be described briefly at this place.

It was possible, in the locality where the work was carried on, to obtain feces from individuals heavily infested with hookworms in sufficient quantity for all purposes, therefore the chief problem which presented itself was the finding of a satisfactory method of culture. At first the cultures were all made with animal charcoal (bone-black), according to Looss's method, in Petri dishes or large soup plates. This method proved unsatisfactory. The culture mass was invaded by fly larvae, especially those of small fruit flies, and in many cases was spread all around in the dishes. In some of the Petri dishes the wanderings of the fly larvae sealed the dishes and kept out sufficient air for the development of the hookworm larvae. Also, the breaking up of the cultures by the fly larvae, both in the Petri dishes and soup plates, often made it impossible to wash the hookworm larvae out into water, without mixing in quantities of bone-black. Further, the making up of a sufficient number of bone-black cultures to obtain the large numbers of larvae needed in the work proved to be a disagreeable, tedious and time-consuming process. After the work had progressed sufficiently to show the difficulties of the bone-black cultures, one of us (Ackert) devised a much more satisfactory method of obtaining the larvae needed. This method can only be used in connection with some such isolating apparatus as that just described. It is much less time consuming than the bone-black culture, and yielded a much surer supply of hookworm larvae. As a culture medium, soil from the yard back of the laboratory was used. This soil was first heated to a temperature of 150° F., to insure the killing of all nematodes, and was placed to the depth of about two inches in shallow tin pans, eleven inches in diameter, with holes punched in their bottoms to permit drainage. To make the culture the stool was spread out in a layer about one fourth of an inch in thickness on the surface of the soil in the pan and covered with about 1 mm. of wood or charcoal ashes. The sprinkling of ashes over the stool inhibits the growth of mould and also prevents the formation of a hard crust. These cultures should be kept in a screened place, protected from rats and other animals, and must be kept well moistened. Under the conditions in Trinidad the hookworm larvae reach maturity in five days. To isolate the mature larvae the top one half inch of the culture is scraped off and placed in the isolating apparatus. Under tropical conditions only feces which have not become contaminated with free-living nematodes are satisfactory for culture purposes, because, if the free-living nematodes get started in the cultures, they develop with such rapidity that the hookworm larvae either fail to develop in large numbers or are difficult to use. The soil-ashes method of culturing, if rightly handled, will yield an abundance of hookworm larvae. In one month we made 26 of these cultures which yielded a total of over 36,500 mature hookworm larvae. Four cultures yielded more than 3,000 larvae each. Six of the twenty-six cultures were unsatisfactory, producing less than 200 larvae. This method was certainly employed much more advantageously than the animal-charcoal culture method, since it took much less time to prepare the material and yielded a much higher percentage of successful cultures.

STANDARDIZATION EXPERIMENTS ON THE ISOLATING APPARATUS.

Baermann (1917 a) performed a series of thirteen experiments to test the accuracy of the isolating apparatus which he devised. In his early experiments he used a sieve 7.5 cm. in diamter and 3.5 cm. in height, placed in a small funnel, and studied the results obtained in attempting to isolate larvae from small amounts of soil (50 cc. to 100 cc.). He later used a much larger sieve and funnel for the examination of the samples taken from the field with, as he states, uniformly satisfactory results (Baermann, 1917 b). The results of his experiments with the small apparatus were very satisfactory. In one case he was able to recover seven out of eight larvae placed in the soil. Baermann further found that the mature hookworm larvae travel through the loose soil in the sieves at the rate of 1 cm. in two minutes, and that they fall through the water in the funnel at the rate of

1 cm. per second. In one of his experiments the larvae began to appear at the bottom of the rubber tube in three minutes after the experiment was set up, and the majority were recovered in half an hour. He also found that it was difficult to isolate the larvae from compact coherent soil unless sufficient water was present to immerse the soil and cause it to crumble.

We found it necessary to carry the standardization of the isolating apparatus much farther than Baermann did, and are able to add a number of points to the important findings which he recorded. At the beginning of the work in Trinidad two of us (Cort and Augustine) began a series of experiments to determine the accuracy of the apparatus. Although the apparatus we used conformed as closely as possible to that described by Baermann, and although we had obtained some fairly satisfactory results in Baltimore, the early experiments gave such variable results that they were uniformly unsatisfactory. Finally one of us (Payne) attempted to stimulate the larvae in the soil samples to greater activity by the use of warm water. The higher percentage of larvae which she obtained led to experimentation along this line with satisfactory results. We have since found that when the temperature of the water used in the funnels is lower or only slightly above that of the soil the percentage of larvae isolated is very low (see Table I). This explains the unsatisfactory results of our first experiment, since the water was often used directly from a cistern and was about 2° F. below the temperature of the soil. Baermann makes no mention of the temperature of the water used in his experiments and subsequent soil examinations. Our results indicate that he must have been using water appreciably warmer than the soil to obtain the satisfactory results which he reported.

We will record here the results of the experiments performed, after the apparatus was working satisfactorily, to test the influence of the temperature of the water, the type of soil and the degree of moisture of the soil on the accuracy of the results. We also performed experiments to test the amount of time needed for the larvae to escape from the soil into the water.

Table I gives the results of a series of experiments performed by two of us (Ackert and Cort) to determine the accuracy of the large isolating apparatus with clay loam soil, with different degrees of moisture and with the water in the funnels at different temperatures. The soil used was taken near our laboratory and was the common type found in cultivated areas in the part of Trinidad where we were working. The technic of handling the apparatus in these experiments was the same as that described earlier in this paper. The amount of dirt used was one pint, which was broken up uniformly and spread out evenly in the sieves. The larvae were counted on slides and washed onto the upper surface of the soil in the sieves. This necessitated their migration through the whole depth of the soil in the sieves, making the conditions more difficult than in the samples taken from the field. The soil was either moist or saturated. The temperature of the water was taken just before the sieves were placed in the funnels. Several significant points are brought out by Table I. In the first place it shows that under favorable conditions with the large isolating apparatus at least 50 per cent. of the larvae present will be regularly recovered. Further, it is clearly shown that to obtain satisfactory results the temperature of the water must be at least 10° F. higher than that of the soil (Table I, *c* and *d*). In the next place an examination

TABLE I.

Effect of temperature and moisture on percentage of recoveries in clay loam.

(a) Temperature of soil 82°–84° F. Temperature of water 120° F.

Moist.				Saturated.			
Test No.	No. larvae put in.	No. larvae recovered.	Percentage recovered.	Test No.	No. larvae put in.	No. larvae recovered.	Percentage recovered.
1....	104	69	66.2	16...	120	35	31.8
2....	122	45	36.8	17...	113	47	41.5
3....	106	41	38.6	18...	185	64	34.6
4....	103	40	38.8	19...	90	50	55.5
5....	112	36	32.1	20...	153	23	15
6....	100	32	32	21...	108	28	25.9
7....	100	45	45	22...	99	40	40.4
8....	117	26	22.2	23...	177	98	55.4
9....	106	70	66	24...	155	68	43.2
10....	104	103	99	25...	168	51	30.3
11....	146	113	77.3	26...	142	30	21.1
12....	118	105	88.5	27...	110	68	61.8
13....	145	73	50.3	28...	176	19	10.7
14....	184	139	59.2	29...	93	7	7.5
15....	130	68	40				
			Av. percentage, 52.8				Av. percentage, 33.9

(b) Temperature of soil 80° F. Temperature of water 90° F.

30....	105	57	54.2	39...	110	46	41.8
31....	111	54	48.6	40...	130	65	50
32....	98	37	37.7	41...	122	64	52.4
33....	94	73	77.6	42...	94	38	40.4
34....	138	58	42	43...	163	93	57
35....	140	64	45.7	44...	135	51	37.7
36....	107	56	52.3	45...	140	76	54.3
37....	148	105	70.9				
38....	124	55	44.3				
			Av. percentage, 52.5				Av. percentage, 47.3

(c) Temperature of soil 84° F. Temperature of water 89° F.

Moist.				Saturated.			
Test No.	No. larvae put in.	No. larvae recovered	Percentage recovered.	Test No.	No. larvae put in.	No. larvae recovered.	Percentage recovered.
46....	105	33	31.4				
47....	108	42	38.8				
48....	90	17	18.8				
49....	98	24	24.4				
50....	111	14	12.6				
51....	90	18	20				
52....	105	17	16.1				
53....	103	17	16.5				
54....	93	6	6.4				
			Av. percentage, 20.5				

(d) Temperature of soil 81° F. Temperature of water 79° F.

55....	115	15	13	64...	129	5	3.8
56....	163	15	9	65...	129	11	8.5
57....	205	38	18.5	66...	261	33	12.6
58....	145	14	9.6	67...	176	10	5.6
59....	131	7	5.4	68...	144	11	7.6
60....	130	10	7.6	69...	256	6	2.3
61....	243	25	10.2	70...	146	16	10.9
62....	95	12	12.7	71...	126	12	9.5
63....	90	8	8.8	72...	173	25	14.4
			Av. percentage, 10.5				Av. percentage, 8.3

of Table I, *a*, *b* and *d* shows that the average percentage of larvae isolated was constantly less from saturated than from moist soil. This point is also shown in Table V and in Table II where it can be seen that the percentage from saturated clay was uniformly low.

TABLE II.

PERCENTAGE OF RECOVERIES FROM DIFFERENT TYPES OF SOIL.

Test No.	Type of apparatus used.	Amt. of soil.	Types of soil.	Moist. cont. of soil.	Tempt. of soil.	Tempt. of water.	No. larvae put in.	No. larvae recovered.	Percentage of recoveries.
1...	Large	1 pt.	Cl. Loam	Moist	Rm. Tempt.	115° F.	81	62	76.5
2...	"	"	"	"	"	"	46	25	54.3
3...	"	"	"	"	"	"	54	47	89.2
4...	"	"	"	"	"	"	73	52	71.2
5...	"	"	"	"	"	"	61	45	73.6
6...	"	"	Sand	"	"	"	49	30	61.2
7...	"	"	"	"	"	"	67	14	20.8
8...	"	"	"	"	"	"	83	46	55.4
9...	"	"	"	"	"	"	51	29	56.8
10...	"	"	"	"	"	"	71	46	66.1
11...	"	"	Cl. Sand	"	"	"	40	0	0
12...	"	"	"	"	"	"	50	4	8
13...	"	"	"	"	"	"	51	11	21.5
14...	"	"	"	"	"	"	50	2	4
15...	"	"	"	"	"	"	74	13	17.5
16...	Small	2 oz.	Cl. Loam	"	85° F.	116° F.	100	80	80
17...	"	"	"	"	"	"	91	81	89
18...	"	"	Sand	"	"	"	136	130	95.5
19...	"	"	"	"	"	"	143	119	83.2
20...	Large	1 pt.	Cl. Loam	"	Rm. Tempt.	115° F.	150	128	85.3
21...	"	"	"	"	"	"	146	81	55.4
22...	"	½ pt.	Clay	Saturated	"	"	125	30	24
23...	"	"	"	"	"	"	149	54	38
24...	"	"	"	"	"	"	100	21	21
25...	"	"	"	"	"	"	137	25	19
26...	"	"	"	"	"	"	130	46	35
27...	"	"	"	"	"	"	139	32	23
28...	"	"	"	"	"	"	127	31	26
29...	"	1 pt.	"	"	"	"	550	220	40
30...	"	"	Humus	Moist	"	"	250	125	50

Table II shows the effect of different types of soil on the percentage of larvae isolated. Test numbers 1 to 15 are the results of an experiment (by Augustine) to compare the percentage of recoveries in three different types of soil, treated in exactly the same way, and using larvae from the same culture. The clay loam was the same soil used in Table I, the sand was a coarse red sand and the clay sand was a very finely divided sand and clay mixture. The low percentage of recoveries from the clay sand indicates that when the particles of soil are very fine, so that they pack closely together, the escape of the larvae into the water is inhibited. Test numbers 10 to 19 of Table II also give a comparison of the recoveries from clay loam and sand obtained in a different series of experiments (by Augustine). The percentage of larvae isolated here is very high, perhaps because only 2 oz. of soil were used, but no differences can be noted between the clay loam and the sand, which in this case was also coarse. Test numbers 20 to 30 of Table II were controls used in the course of examinations from the field (by Cort). The low percentage of recoveries from saturated clay supports the view expressed in regard to the clay sand, because the particles of the clay were so finely divided that, when saturated, they became closely packed, reducing greatly the spaces in the soil. That the texture of the soil is an important factor in the percentage of larvae which can be isolated is shown clearly by the series of experiments (by Payne) in Table III. This table shows that the mixing of coarse gravel with soil increases very appreciably the percentage of larvae that can be isolated from it, and indicates that, when dealing with very finely divided soil in field samples, the mixing in of sterilized coarse soil or gravel would be advantageous

TABLE III.

THE EFFECT OF MIXING COARSE GRAVEL WITH SOIL ON THE PERCENTAGE OF LARVAE RECOVERED.

Type of soil.	Amount of soil.	Numbers of hookworm larvae put in.	Number recovered.	Percentage of recoveries.
Clay Loam packed into a solid ball	Ball 1½" in diameter	20	4	20
Clay Loam packed into a solid ball	Ball 1½" in diameter	47	5	10.6
Clay Loam packed into a solid ball, but mixed with gravel.	Ball 1½" in diameter	59	55	93
Clay Loam packed into a solid ball, but mixed with gravel.	Ball 1½" in diameter	39	39	100
Clay Loam mixed with gravel.	¼ pint	186	151	81

In Table IV are given the results of the isolation of larvae from a series of migration experiments (by Augustine). These tests differ from those previously recorded in that the larvae had lived in the soil for periods varying from 15 hours to 11 days before being placed in the apparatus. The higher percentage of larvae recovered in these experiments is probably due both to the use of smaller quantities of soil and to the fact that the larvae had a shorter distance to migrate. In some of these cases it was known that the hookworm larvae were on the surface, and, therefore, this part of the soil sample was put into the sieve first. This shows that in experimental work it may be possible to increase the percentage of larvae which can be isolated by careful handling of the soil.

TABLE IV.

PERCENTAGE OF RECOVERIES OF LARVAE WHICH HAD BEEN IN SOIL FROM 15 HRS. TO 11 DAYS. SOIL USED: MOIST CLAY LOAM. SOIL AT ROOM TEMPERATURE. WATER 118° F.

Test No.	Time larvae had lived in soil.	No. larvae put in soil.	No. larvae recov. with small appr.	No. larvae recov. with large appar.	Total No. larvae recov.	Percentage.
1....	15 hours	800	409	115	524	66.7
2....	15 "	1,000 (?)	583	275	858	85.8
3....	15 "	1,000 (?)	460	406	866	86.6
4....	15 "	525	363	69	432	81.9
5....	2 days	300	248		248	82.6
6....	2 "	300	289		289	96.3
7....	3 "	100	87		87	87.0
8....	4 "	300	217		217	72.3
9....	4 "	300	259		259	86.3
10....	6 "	100	52		52	52.0
11....	9 "	100	51		51	51.0
12....	11 "	512	89	148	237	46.4

The experiments shown in Table V (by Payne) give an idea of the length of time it takes for larvae to pass through the isolating apparatus. The data in this table show that, while the great majority of the larvae which can be recovered pass into the water within six hours, there is an appreciable number which can be recovered only after a longer period. It is significant to note that a larger percentage of the larvae come out of saturated soil between six and twenty-four hours than from the moist soil. It is possible that if the saturated soil were left for a still longer period, the percentage of larvae isolated would be increased.

TABLE V.

EXPERIMENT TO TEST THE TIME OF PASSAGE OF LARVAE THROUGH ISOLATING APPARATUS. SOIL: CLAY LOAM AT ROOM TEMP. WATER AT 115° F.

A. Soil moist.

Test No.	Type of apparatus.	Amt. of soil.	Larvae put in.	Larvae recovered after 1 hr. No.	%	3 hrs. No.	%	6 hrs. No.	%	24 hrs. No.	%	Total No.	Percentage.
1.....	large	1 pt.	140	12	8.6	34	24.2	17	12.1	6	4.3	69	49.2
2.....	small	½ pt.	113	29	25.7	35	30.9	21	18.6	5	4.4	90	79.6
3.....	"	¼ pt.	106	19	17.9	23	21.7	15	14.1	2	1.9	59	55.6
4.....	"	¼ pt.	83	24	28.9	36	43.4	10	12	3	3.6	73	87.9

B. Soil saturated.

Test No.	Type of apparatus.	Amt. of soil.	Larvae put in.	Larvae recovered after 1 hr. No.	%	3 hrs. No.	%	6 hrs. No.	%	24 hrs. No.	%	Total No.	Percentage.
5.....	large	1 pt.	124	8	6.4	17	13.7	6	4.8	17	13.7	48	38.6
6.....	small	½ pt.	105	3	2.8	19	18.1	9	8.6	15	14.3	46	43.8
7.....	"	¼ pt.	111	1	0.9	15	13.5	6	5.4	17	15.3	39	35.1
8.....	"	¼ pt.	93	8	8.6	12	12.9	10	10.8	12	12.9	42	45.2

The series of experiments just described serves as a control on the examination of soil samples from the field where the number of larvae was unknown, gives some idea of the limitations of the isolating apparatus, and indicates in a general way the method by which it works. A number of points are still not clear. Just what causes the larvae to come out of the soil into the water is still unknown. If it were an attraction to moisture they would come out into water of about the same temperature as the soil as readily as into warmer water and would make their escape quickly, before the whole mass of the soil becomes uniformly moistened. That the escape is a direct reaction to the higher temperature of the water seems impossible, because in a very short time the whole soil in the sieve becomes of the same temperature as the water, and even after six hours a number of larvae will escape. Since the percentage of larvae which can be isolated is much greater in porous than in very compact soil, it is possible as suggested by one of us (Payne) that the larvae, stimulated to activity by the warmth and aided by the currents, escape by falling through the water-filled spaces of the soil. Be that as it may, the important point is that the apparatus really works, and makes it possible to isolate a considerable percentage of the larvae present, even from a rather large volume of soil.

USES OF THE ISOLATING APPARATUS IN INVESTIGATIONS AND HOOKWORM CONTROL WORK.

The apparatus which has just been described was utilized in every phase of the researches carried out in Trinidad during the past summer. In the field investigations its use made possible the examination of large numbers of soil samples from various situations, in areas where hookworm disease was prevalent, to determine the exact sources of infestation of the people. It also made it possible to follow the reduction of soil infectivity after the elimination of soil pollution. In the experimental work it was used in the study of the conditions under which the hookworm eggs can develop, and has made it possible to determine the extent of the migrations of the larvae in different situations. It was also utilized in studying the rate of unsheathing and the length of time the larvae can live in different soils. The uses made of this apparatus by Baermann and by our expedition suggest a wide range of possibilities in the study of hookworm larvae, both in the field and in laboratory experimentation. Further, it might be used in the study of free-living nematodes, and might be of practical help in investigations on the soil stages of nematodes parasitic on plants. The use of the isolating apparatus will also make possible advances in the knowledge of all parasitic nematodes which have a free-living stage, such as Strongyloides stercoralis and the Strongylidae of domesticated animals. Aside from the value of this apparatus in investigations, it can be successfully utilized, as shown by Baermann's work, in connection with campaigns for the control of hookworm disease, in locating and evaluating the sources of infestation of the people, and in discovering the sources of reinfestation after the completion of a piece of work.

SUMMARY.

1. The apparatus for isolating hookworm larvae from soil, described in this paper and shown in Figs. 1 and 2, which is similar to an apparatus devised by Baermann, makes it possible to isolate hookworm larvae from considerable quantities of soil.

2. It is possible to distinguish mature hookworm larvae, both sheathed and unsheathed, from other nematodes found in soil, by their characteristic structure and movement.

3. For experiments with the isolating apparatus mature hookworm larvae were obtained by spreading feces containing hookworm eggs on soil in large pans and covering this with a thin layer of ashes.

4. Experiments to standardize the isolating apparatus showed (1) that to isolate a satisfactory percentage of the larvae the water must be at least 10° F. warmer than the soil, (2) that a slightly higher percentage of larvae can be isolated from moist than saturated soil, (3) that in soil with very finely divided particles the percentage of larvae which can be isolated is less than in coarser soils, and (4) that while most of the larvae escape from the soil into the water, in the first six hours, an appreciable number come out after this time.

5. It is probable that the passage of the larvae from the soil to the water is not a reaction to moisture or a higher temperature, but is brought about by the active larvae falling through spaces in the soil until they reach the water.

6. The isolating apparatus makes possible the study of soil nematodes, both free living and the larvae of parasitic species, in their natural environment, and can be utilized in investigating the sources of human infestation in hookworm control work.

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INVESTIGATIONS ON THE CONTROL OF HOOKWORM DISEASE.

III. A Discussion of the Finding of Unsheathed Hookworm Larvae in the Soil.*

By WILLIAM W. CORT, Ph.D., DONALD L. AUGUSTINE, B.S., JAMES E. ACKERT, Ph.D., FLORENCE KING PAYNE, M.D., and GEORGE C. PAYNE, M.D.

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Looss (1911, p. 429) makes the following statement in his monograph on the hookworm: "During the rest of their free existence, the mature larvae are surrounded by the detached skin of the second larval stage, which acts as a protective envelope. It is thrown off in the normal course of events when the worms pass into a suitable host and thus enter the third stage of their life." These two sentences sum up the conception which dominates our present ideas of the life of infective hookworm larvae. In fact the terms "encysted," "encapsuled" or "sheathed" larvae are commonly used as synonyms of infective larvae. Accepting this conception, we were surprised to find that it is a common occurrence for mature hookworm larvae to loose their sheaths in soil, and that such unsheathed larvae are able to continue their lives and are probably infective. On account of the far-reaching significance of this finding and because it forms a part ot the data and conclusions of the investigations of all the members of our expedition, we decided to publish the present paper jointly.

The fact that infective hookworm larvae may become unsheathed under certain conditions was first noted by Leichtenstern (1887). Other authors who reported this phenomenon were Lambinet (1902), Herman (1905), and Looss (1911). As Looss (1911, pp. 429–436) has discussed in detail the findings of these authors, it will not be necessary to consider them further here. All of these invesigators were so influenced by the idea that the infective hookworm larvae live normally enclosed in sheaths that they considered their findings as representing abnormal conditions. More recently Baermann (1917, 624) noted the presence of unsheathed forms amongst larvae which he isolated from samples of soil. He apparently experienced difficulty in identifying such unsheathed larvae as young hookworms, and ascribed the loss of the sheath to the effect of the passage through the isolating apparatus, which he used.

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* This paper is the third of the series on the hookworm investigations by the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University, carried on with the cooperation of the International Health Board, Rockefeller Foundation.

The first intimation that infective hookworm larvae might loose their sheaths in soil came early in our work, when one of us (Augustine) found in one of his experiments that some larvae which had been placed in the soil came to the surface without their sheaths. Following this discovery similar findings were made in quick succession in all of our experiments and in the samples from the field. The data given below, taken from our studies in Trinidad, show that a large percentage of hookworm larvae complete their second moult in the soil and live in the unsheathed condition.

In connection with some studies (by Cort and Payne) which will be included as later numbers of this series, a large number of samples of soil were examined for hookworm larvae. In a series of samples taken from a cacao grove, where concentrated centers of soil pollution occurred, the sheathed and unsheathed larvae of twenty-four samples were counted. Out of a total of 2,159 infective hookworm larvae isolated from these samples, 1,806 were sheathed and 353 unsheathed, or 83.6 per cent. sheathed and 16.4 per cent. unsheathed. The considerably higher percentage of sheathed larvae found in this series is partly explained by the findings from one soil sample, from which was obtained a total of 1,802 sheathed and 28 unsheathed hookworm larvae.

Much more striking were the findings from a large series of soil samples taken from a highly polluted area in a sugar cane field. In 108 positive soil samples from this area, the number of infective hookworm larvae per sample varied from 1 to 937. Out of a total of 4,265 hookworm larvae isolated from these samples, 1,793 were sheathed and 2,471 unsheathed or 42 per cent. sheathed and 58 per cent. unsheathed. These soil samples were taken from two types of localities, the first type at regular intervals along the drains, and the second between the rows of cane, at places where pollution was known to have occurred. Although stools were occasionally found in the drains, the samples were not taken at definite polluted spots as were those from between the rows, and they were therefore not so directly related to the points of pollution. Further the small numbers of larvae obtained from the drain samples and their almost uniform distribution along the drains suggested that these larvae were some distance from the point of development. With this in mind, it is interesting to make a comparison between the percentages of unsheathed larvae from these two situations. In the drains, in 39 positive samples, there were found 698 mature hookworm larvae of which 135 were sheathed and 563 unsheathed or 19.3 per cent. sheathed and 80.7 per cent. unsheathed. From the 69 positive samples taken from polluted spots between the rows 3,567 mature hookworm larvae were isolated. Of these 1,658 were sheathed and 1,909 unsheathed, or 46.5 per cent. sheathed and 53.5 per cent. unsheathed. The higher percentage of unsheathed larvae in the drains may perhaps be related to the fact noted above that the samples were not taken at centers of pollution, and therefore that larvae were probably farther away from the places where they developed, and perhaps on the average older than those taken from the other situation.

TABLE I.

Data on unsheathing of hookworm larvae in migration experiments.

No. of experiment.	Original number of sheat'd larvae.	Age of culture from which larvae were obtained.	Type of soil used.	Time that larvae remained in soil.	Number of sheat'd larvae recovered.	Number of unsheat'd larvae recovered.	Percentage of sheat'd larvae recovered.	Percentage of unsheat'd larvae recovered.
1....	1,000	5 days	Moist black clay loam	15 hrs.	164	702	18.9	81.1
2....	512	5 days	Moist black clay loam	11 days	3	234	1.2	98.8
3....	525	5–6 days	Moist red clay loam	15 hrs.	36	396	8.3	91.7
4....	1,300	8 days	Moist sand	7 days	43	332	11.4	88.6
5....	1,300	8 days	Saturated sand	8 days	57	800	6.6	93.4
6....	1,000	7 days	Moist black clay loam	7 days	180	141	56.0	44.0
7....	1,000	7 days	Moist black clay loam	7 days	245	272	47.3	52.7

In connection with a series of experiments on the migration of mature hookworm larvae in various types of soil (by Augustine) some interesting data were obtained on the loss of the sheath. In these experiments sheathed larvae, five to eight days old, were placed in an area one inch in diameter at the center of the surface of soil which was spread out to the depth of 1.5 cm. in thirteen-inch pans.

This soil was later divided into zones and placed in the isolating apparatus, for the removal of the larvae. The complete results of these and similar experiments on migration will be given in a later number of this series. In the present paper we are interested in the fact that young sheathed larvae were placed in these pans, and after periods of time varying from 15 hours to 11 days a high percentage of unsheathed larvae was recovered. Table I gives a summary of these results.

These experiments were performed in the laboratory, the surface of the soil in the pans being sprinkled every day. It will be seen from Table I that a high percentage of unsheathed larvae was obtained, even when the matured hookworm larvae had been in the soil only 15 hours. Since different types of soil were used this series indicates that the loss of the sheath does not depend on any particular type of soil. In connection with these experiments microscopic examinations were made directly of small particles of soil from the centers, where the larvae had been placed. On one piece of clay loam two active unsheathed hookworm larvae were found and one empty sheath, which had its anterior part imbedded in soil. Another direct examination was made of a small particle of sand. On this were found six active unsheathed larvae and three empty sheaths.

Another series of experiments was carried out by one of us (Ackert), giving further evidence on the unsheathing of the mature hookworm larvae. These experiments which will be described fully in a later number of this series were undertaken to study the conditions under which hookworm eggs will hatch and develop. Portions of fresh stools from individuals heavily infested with hookworms were placed in soil in flower pots. These flower pots were then placed out of doors, where they were subjected both to sun and shade, and wetting from rains followed by partial drying. Table II includes the data from these experiments which relate to the unsheathing of the larvae.

The conditions in these experiments are more like those in nature than in the former series, since in every case feces containing hookworm eggs were used. The large number of unsheathed larvae which were isolated add to the evidence that the loss of the sheath is a common phenomenon. A comparison of the experiments numbered XXVI, XXVII, XXVIII and XXIX is very interesting. In these experiments the conditions were the same, except that the last two were kept four days longer than the others. The additional time was

TABLE II.

Data on percentage of sheathed and unsheathed larvae found in series of flower pot experiments.

No.	Soil.	Conditions of experiment.	Conditions of light.	Days out.	Sheathed.	Un-sheathed.	Percent-age sheathed.	Percent-age un-sheathed.
1 ..	Clay loam	Buried ½ inch	Sun and shade	29 days	0	33	0.0	100.0
V ..	Clay loam	Buried ½ inch	Shade and sun	21 days	52	30	63.4	36.6
XVI ..	On clay loam	Covered 8 mm.: with ashes	Shade and sun	23 days	420	885	32.1	67.9
XVIII ..	Clay loam	Buried ½ inch	Shade	25 days	1,861	0	100.0	0
XIX ..	Clay loam	Buried ½ inch	Shade and sun	27 days	139	30	82.2	17.8
XXVI ..	Clay loam	On top	Shade and sun	13 days	3,250	0	100.0	0
XXVII ..	Clay	On top	Shade and sun	13 days	1,112	0	100.0	0
XXVIII ..	Clay loam and clay	On top	Shade and sun	17 days	53	400	11.2	88.8
XXIX ..	Clay loam	On top	Shade and sun	17 days	145	1,564	8.4	91.6
XXX ..	Clay loam	On top	Light and shade	18 days	13	119	9.8	90.2
XXXV ..	On clay loam	Covered 3 mm.: in ashes	Shade	23 days	2,250	720	75.5	24.5
XXXVI ..	Ashes	Buried 3 mm.	Shade	23 days	525	508	50.8	49.2
XXXVII ..	On clay loam	Buried 8 mm.	Shade	23 days	2,469	0	100.0	0
XL ..	On clay loam	Buried 8 mm. in ashes	Sun and shade	15 days	108	161	40.1	59.9
XLI ..	Clay loam	On surface	Sun and shade	15 days	10	1,290	.7	99.3
XLII ..	Clay loam	Buried ½ inch	Sun and shade	14 days	1,304	971	57.3	42.7

apparently the factor which caused a very rapid unsheathing. A further analysis of the table shows that no difference can be found in the percentages of unsheathed larvae between those experiments in which the fecal material was placed on the surface of the soil and those in which it was slightly buried. As far as the data go they seem to indicate that when the flower pots were exposed to alternating sunlight and shade there was a greater degree of unsheathing than when they were kept in the shade. A comparison of experiments XVIII, XXXV, XXXVI and XXXVII, which were kept in the shade, with others, in which the conditions were the same except for alternating sunlight and shade supports this view. Perhaps the alternating sunlight and shade with the partial drying of the soil stimulated the larvae to activities, which resulted in the greater degree of unsheathing.

In a series of experiments performed by one of us (Florence King Payne), to test the distance which hookworm larvae can migrate, when buried in soil, additional data were obtained on the unsheathing of the larvae. Sheathed larvae were placed at the bottom of columns of soil of various heights, and later this soil was divided into zones, placed in the isolating apparatus, and the hookworm larvae obtained counted. The soil was a black clay loam. Table III shows the percentage of sheathed and unsheathed larvae recovered in this series of experiments.

It is shown in Table III that when sheathed larvae are buried in soil for periods varying from two to ten days, a considerable percentage of them lose their sheaths. Further, under the conditions of these experiments the percentage of unsheathed larvae increased the

TABLE III.

Findings of hookworm larvae from soil in vertical migration experiments.

No. of days larvae had remained in soil.	Total number recovered.	Number sheathed.	Number unsheathed.	Percentage sheathed.	Percentage unsheathed.
2 days........	256	129	127	50.4	49.6
3 days........	26	12	14	46.0	54.0
4 days........	1,505	767	738	50.8	49.2
6 days........	14	4	10	28.5	71.5
7 days........	418	126	292	30.1	69.9
8 days........	271	56	215	20.6	79.4
9 days........	34	17	17	50.0	50.0
10 days........	30	3	27	10.0	90.0

longer the larvae remained in the soil. Another important point was shown in one of the experiments when, after four days, 188 larvae were recovered which had migrated vertically between 5 and 6 inches. Of these larvae 140 were sheathed and 48 unsheathed, or 74.5 per cent. sheathed and 25.5 per cent. unsheathed. This observation is significant since it shows that the larvae do not necessarily lose their sheaths while migrating through clay loam soil.

The data given above show that under the conditions in Trinidad, during the months of June, July and August, when the daily range of temperature is from about 74° to 94° F., infective hookworm larvae very commonly lose their sheaths in soil and continue to live actively.

It is a well-known fact that the mature hookworm larvae are infective while still enclosed in their sheaths, but that for penetration into their host they must break out of their sheaths leaving them on the surface of the skin. It would seem, therefore, that active larvae already unsheathed would be able to penetrate the skin more easily and quickly than those which come in contact with the surface of the skin while still enclosed in their sheaths. That unsheathed mature hookworm larvae can penetrate mammalian skin was shown by one of us (Augustine) in an experiment in which unsheathed larvae were observed to penetrate the skin of a rat.

The cause of the loss of sheath and the exact conditions under which it occurs are not clear from the data at hand. Baermann's idea, that the finding of unsheathed hookworm larvae in his examinations was due to the passage through the isolating apparatus, cannot be applied to our findings. We have often found in our controls that some unsheathing does occur in the passage through the soil in the sieves, but in most cases practically all of the larvae will pass through the apparatus without losing their sheaths. It has already been noted that unsheathed larvae were found by direct microscopical examinations of small particles of soil. Further, soil samples taken from the cane-field and treated in exactly the same way show all degrees of variation in the percentage of sheathed and unsheathed larvae, after passage through the isolating apparatus. One sample yielded 1 sheathed and 247 unsheathed, another gave 407 sheathed and no unsheathed, while other samples showed all gradations between these extremes.

Few observations have been made by us which bear on the cause of the loss of the sheath, and the conditions under which this loss occurs. That the unsheathing is not limited to any particular type of soil is shown by the fact that it occurs in clay, clay loam and sand. It also occurs in differing conditions of moisture. That a longer time in the soil seems in some cases to increase the percentage of unsheathed larvae is indicated by some of our experiments, and yet in other cases a very high percentage of unsheathed larvae was obtained after only 15 hours in the soil, and in another experiment 25 days after feces containing eggs had been placed in soil large numbers of sheathed, but no unsheathed larvae were isolated. Certain experiments show that the unsheathing is greater where the soil containing the larvae is exposed to alternating sunlight and shade, than when it is kept constantly in the shade. This might indicate that conditions which increase the activity of the hookworm larvae in the soil tend to bring about the loss of the sheath, but the fact already cited, that larvae which had migrated vertically through 5 to 6 inches of clay loam remained sheathed, shows that they can go through a period of intense activity completely surrounded by soil, and still retain their sheaths. Perhaps further investigations on the rate and degree of unsheathing under different conditions of soil, temperature and moisture will give an explanation of the cause of the unsheathing of the infective hookworm larvae in soil.

As stated earlier in this paper, the finding that the loss of the sheath is a common process in infective hookworm larvae in the soil necessitates a revision of many ideas in regard to this phase of the life-history of the hookworm. Most of the knowledge of the activities of the infective hookworm larvae has come from experiments on sheathed larvae, studied under unnatural conditions, such as in cultures, in water, or before they have left the fecal mass, where they develop. Of what actually occurs in soil little is known, and since it has now been discovered that a very large percentage of hookworm larvae lives in the soil without any protective sheath, it would seem necessary to make investigations of various kinds on the activities and relation to environment of the unsheathed larvae, and also on the larvae as they live in the soil under natural conditions. Such investigations on a comparatively large scale are made possible through the use of the isolating apparatus described in the second number of this series (Cort et al., 1921). That studies of this kind will give some results quite at variance with present ideas is shown by the results of the experiments carried out in Trinidad, which will be given in the succeeding numbers of this series.

SUMMARY.

1. Although various investigators have reported that mature hookworm larvae lose their sheaths under certain conditions, it has been the general opinion that they live normally enclosed in sheaths.

2. It was found that in a series of soil samples examined from places polluted by people infested with hookworms a large proportion of the mature larvae were without sheath.

3. Also, in experiments on the conditions under which hookworm eggs hatch and develop and on the migrations of the infective larvae, it was found that in soil a large proportion of the larvae soon became unsheathed.

4. These findings suggest that it is a common thing for mature hookworm larvae to lose their sheaths and continue to live in soil.

5. Since most of the knowledge of the activities of mature hookworm larvae has come from studies on sheathed forms, it will probably be necessary to revise many of our ideas on the life of infective hookworm larvae in the soil.

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INVESTIGATIONS ON THE CONTROL OF HOOKWORM DISEASE.

IV. The Relation of the Domestic Chicken to the Spread of Hookworm Disease.*

By JAMES E. ACKERT, Ph.D.

(Received for publication, September 20, 1921.)

Introduction.

It has long been known that domestic chickens will disseminate human excrement, and as early as 1910 Dock and Bass (p. 87) ascertained that hookworm eggs can retain their viability and hatch after passing through a chicken's digestive tract. But the proportion of hookworm eggs which produce infective larvae seems to be wholly unknown. As chickens usually abound in regions where hookworm disease is prevalent, it is important to determine whether they are a real factor in the spread of the disease. An opportunity to investigate this problem in Trinidad, British West Indies, where over eighty per cent. of the natives are infested with hookworm, was recently afforded me by the International Health Board, through the efforts of Dr. William W. Cort and Dr. George C. Payne. I wish at this time to acknowledge my indebtedness to them for complete cooperation and many helpful suggestions, and also to my other colleagues, Dr. Florence King Payne, and Mr. D. L. Augustine.

Methods.

For this and other experiments a private dwelling was leased and converted into a field laboratory. As the house was built on piles, very suitable animal quarters were afforded underneath it. The investigations were begun at the close of the dry season, and the clay-loam soil beneath it was found to be wholly free from hookworm larvae and nearly so from free-living nematodes. The experiments were facilitated by the absence from the premises of children and other irresponsible persons who might pollute the soil.

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* This paper is the fourth of the series on the hookworm investigations made by the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University, with the Cooperation of the International Health Board, Rockefeller Foundation. It is Contribution No. 55 from the Department of Zoology, Agricultural Experiment Station, Kansas State Agricultural College.

For the chicken experiments two pens were constructed, a large living pen and a small one for the recovery of feces. The small pen was equipped with a woven wire platform that permitted the feces to drop upon clean paper. To ascertain the length of time required for material to pass through the chicken's digestive tract two filter paper capsules of animal charcoal and feces were given to each fowl. In two hours and forty minutes the first black fecal deposit appeared, and for several hours all droppings showed the animal charcoal. At the end of twenty hours after the feeding, the feces were grayish, showing no trace of the animal charcoal. Consequently, in the quantitative tests the feces were usually recovered for at least twenty hours after the feedings were made.

As chickens carry human excrement on their feet, this source of error in the experiments was avoided by administering the feces containing hookworm eggs to the chickens in filter paper capsules. Thus any hookworm larvae found in the pens could have come only from eggs that had passed through the digestive tracts of the fowls. The recovered chicken feces containing hookworm eggs were cultured in animal charcoal in closed Petri dishes and soup plates, and in previously heated garden soil in open pans, both in the laboratory and out of doors. The garden soil as a culture medium worked very satisfactorily both for human and for chicken feces. The gradual heating up to 150° F. did not oxidize the organic material, but killed all nematodes, both sheathed and unsheathed. The feces containing hookworm eggs were placed on top of such soil, or scratched into the surface, approximating natural conditions. After allowing six to ten days for the hookworm larvae to reach the infective stage the cultures were placed in separate units of the isolation apparatus (Cort et al., 1921) and the hookworm larvae obtained counted. The first tests were made with two adult chickens. Their feces voided over night and examined both by smears and the brine-loop-flotation technique showed no nematode eggs or larvae. The various findings were verified by the use of two young chicks about six weeks old. Their feces likewise were free from nematode eggs and larvae.

CAN HOOKWORM EGGS SWALLOWED BY CHICKENS PRODUCE INFECTIVE LARVAE?

A preliminary series of experiments confirmed the early work of Dock and Bass already cited, viz.: that hookworm eggs can retain their viability and hatch after passing through the digestive tract of the chicken. However, the critical test of the chicken as a factor in the spread of hookworm disease is not whether hookworm eggs can remain viable in passing through its alimentary canal and then hatch, but whether these larvae can develop to the infective stage.

To decide this point, a series of feedings was made, the essential features of which are shown in Table I. For this and subsequent feedings the stools of one individual, known as Beggar No. 2, were utilized because of the uniformly large number of hookworm eggs present and the comparative lack of other nematode eggs and larvae. The "cubic inches" of stool administered are approximate rather than accurate, nine filter paper capsules being about equal to one cubic inch. Only sufficient animal charcoal was mixed with the fresh stool to darken it. After recovery the chicken feces were mixed with an equal amount of animal charcoal and cultured in closed Petri dishes and covered soup plates. As shown in Table I, the subsequent examinations revealed infective hookworm larvae varying in the different cultures from one to forty-six.

TABLE I.

Development of infective hookworm larvae in chicken feces and animal charcoal.

Date.	Cubic inches of stool fed.	Feces recovered hrs. after feeding.	Culture medium.	Days cultured.	No. infective larvae recovered.
May 21st....	2	29	Animal charcoal	12	13
May 30th...	1½	22	Animal charcoal	12–15	46
June 1st.....	½	5½	Animal charcoal	10–15	13
June 11th...	¾	5½	Animal charcoal	11–25	1

As animal charcoal is known to inhibit putrefaction of the stool it was thought that the larvae might be able to develop to the infective stage in a culture medium of fowl feces and charcoal and not be able to do so in chicken feces and soil. This, however, proved not to be true, as seven sheathed hookworm larvae were obtained from a culture of fowl excrement and previously heated soil.

Again, it might happen that larvae could mature in such a medium in closed Petri dishes, but fail to do so out of doors in the absence of closed containers kept in the shade. But this doubt was likewise removed when, after a feeding, sixty infective hookworm larvae were taken from a culture of chicken feces and previously heated soil which was placed out of doors in the sun and rain.

WHAT PROPORTION OF THE HOOKWORM EGGS SWALLOWED BY CHICKENS PRODUCE INFECTIVE LARVAE?

An examination of Table I and of the two tests just described reveals the fact that in none of the cultures of chicken feces has a large number of infective hookworm larvae developed. This observation led to an attempt to make some quantitative tests of the proportionate number of larvae developing to the infective stage in such cultures. In these tests the fresh stools of Beggar No. 2 were divided into two very nearly equal portions, one half being cultured directly and the other half given to the chickens. After several hours the chicken feces containing the hookworm eggs were recovered, cultured, and in all respects kept under conditions similar to those of the corresponding human culture. These conditions included culturing in Petri dishes and in earthen pans in the laboratory, and in open containers out of doors. In Table II are given the principal data obtained in the tests. The finding of only one hookworm larva in the test of June 11 might be explained by the fact that the chicken feces were recovered for only 5 hours and a half after feeding, although before the end of this time all of the blackened part of the feces had been voided. The feces from the June 12 test were mixed with previously heated soil and placed in eleven-inch tin pans whose bottoms had been perforated for drainage. These cultures were kept in the laboratory except during the almost daily shower, when they were set out until thoroughly soaked. Thus they were in the sun but a few minutes at a time, immediately after showers. As no animal charcoal was used in administering the feces, and ample time was allowed for the passage of the hookworm eggs through the chickens, this seems to have been a very fair test.

The test of June 18 was similar to the one just described, except that the feces in each case were slightly scratched into the surface of the soil and the cultures set out of doors, where they received approximately four hours of sunshine and a heavy or light shower almost

TABLE II.

Effect of chicken on hookworm development.

Date.	Cubic inches of stools.	Disposal of stool.	Feces recovered hrs. after feeding.	Culture medium.	Environment.	Days cultured.	Infective hookworm larvae obtained.	Percent. of larvae from chickens.
June 11..	1½	Half cultured		Animal charcoal	Laboratory	11	139	
		Half to chickens	5½	Animal charcoal	Laboratory	11-25	1	.7
June 12..	3	Half cultured		Heated soil	Shade	11	4,780	
		Half to chickens	17	Heated soil	Shade	10	215	4.2
June 18..	2	Half cultured		Heated soil	Shade	9	1,744	
		Half to chickens	23	Heated soil	Shade	8	58	3.3
June 30..	3	Half cultured		Heated soil	Sun and shade	9	2,802	
		Half to chickens	22	Heated soil	Sun and shade	8	88 (264)	9.4
July 18..	3	Half cultured		Heated soil	Shade	13	3,180	
		Half to chickens	21	Heated soil	Shade	12	9	.3
July 28..	2	Half cultured		Heated soil	Shade	9	882	
		Half to chickens	25	Heated soil	Shade	8	138	15.6
Aug. 4..	2	Half cultured		Heated soil	Shade	9	925	
		Half to chickens	23	Heated soil	Shade	8	54	5.8

daily. This test represented a close approximation of natural conditions. The next test, June 30, differed slightly in that the feces were simply placed on top of the previously heated soil and then set out of doors. The combined action of fly larvae and rain caused a complete disappearance of the human portion during the third day, while the more fibrous fowl feces were in evidence throughout the eight-day period. In recovering the infective larvae from the chicken culture, the centrifuge tube containing the larvae was emptied after about one third of the fluid was examined. However, as the fluid and larvae had been thoroughly mixed just before the first third was withdrawn for examination, it is probable that the 88 larvae counted represent approximately one third of the number 264.

In the tests of July 18 and 28 extreme care was exercised in making an equal division of the human stool. The feces were placed on top of the culture soil, but the pans were kept in a small closed yard house and sprinkled frequently, avoiding the heavy rains and the direct rays of the sun. But these precautions did not raise the percentage of infective hookworm larvae developing in the chicken feces. Immediately before and after the feeding of August 4, four earthworms and some whole sweet milk were given to one of the chickens to ascertain if a higher protein diet might affect the number of hookworm larvae developing to the infective stage. However, it is seen (Table II) that this had no appreciable effect, the number maturing being 5.8 per cent. of the number attaining the infective stage in the corresponding human culture. These tests indicate that hookworm eggs swallowed by chickens are much less likely to produce infective hookworm larvae than are the eggs in human stools deposited out of doors. As a whole, the tests indicate that less than ten per cent. of the eggs ingested by chickens produce mature hookworm larvae.

THEORETICAL CONSIDERATIONS.

The results from the tests shown in Table II must not be considered exact as several possible sources of error are involved. In the first place, the division of the stool into halves was made optically. But a difference in volume of five per cent. can readily be detected, so that the error here was certainly small. Next, the eggs may not have been evenly distributed through the stool. The only checks on this point were the frequent microscopic examinations of Beggar No. 2's stool, every smear of which showed hookworm eggs. In the third place, any eggs that happened to pass into the caeca on their way through the intestine would probably be delayed too long to be recovered. But this number would not be large. Then, of course, there is the possible error in counting. But after allowing a wide margin for these possible sources of error, it is evident that the great majority of hookworm eggs swallowed by chickens fail to produce infective hookworm larvae.

The cause of this phenomenon has not been definitely determined. However, a possible factor in the reduction is the action of the gizzard on the eggs. This seemed plausible when the writer learned of an experience Dr. Florence King Payne had with *Ascaris* eggs. In an attempt to separate the flocculent albumen from the eggs, small glass beads, two millimeters in diameter, were agitated in the culture dish, with the result that some of the eggs were broken. When eggs with shells as thick as those of *Ascaris* are broken by the concussion resulting from the shaking of small glass beads, it is readily conceivable that the thin-shelled hookworm eggs might suffer much injury in passing through the grit-filled muscular gizzard of the chicken. Especially might eggs be crushed that got into positions where the grit was fine and the muscular contractions severe. The only direct evidence on this point in the experiments was afforded by the difficulty of finding hookworm eggs in the chicken feces. When eggs were readily found in smears of the blackened human stool, it was surprising when six smears of the black chicken feces were examined without finding a single hookworm egg, although infective larvae developed in the culture. In view of these considerations, it seems possible that a part of the reduction in mature hookworm larvae occurring in the cultures of chicken feces and soil may result from injury to the hookworm eggs in the fowl's gizzard.

The inclusion of the urine with the chicken feces probably introduced an unfavorable factor in the production of larvae, as Minagawa (1917) found that the hatching of the hookworm eggs was greatly inhibited in latrines where the urine was mixed with the human excrement. In this author's opinion the failure of the eggs to hatch was due to differences in osmotic pressure caused by the presence of urine in the feces. To obviate this possible factor in the present experiments, the cultures from the tests of June 12, 18, and 30 were placed out of doors, where they were thoroughly washed by the rains nearly every day. The numbers of larvae maturing in these chicken cultures were larger, on the average, than those from such cultures kept indoors and lightly sprinkled, as was done with the cultures from the last three tests (Table II). The evidence here favors the urine hypothesis, but the eggs which produced the 138 larvae from the July 28 test probably hatched in the presence of more urine than did those which gave the 58 larvae from the June 18 test, as the former culture was only gently moistened by the aid of a pipette. However, in either case, it is probable that enough of the urine remained to affect the hatching of some of the eggs.

As the experimental chickens were kept under conditions which greatly reduced the amount of insect food, it might appear that their somewhat limited diet was responsible for the small numbers of hookworm larvae maturing in the feces, but the percentage of infective larvae did not increase when earthworms and milk were added to the rations which included corn, oats, and green grass.

A possible reducing factor may result from certain physiological and morphological differences between human beings and fowls. The greater avian oxygenation, higher temperature and different digestive apparatus, including a cloaca, may form a mixture of metabolic and egestible products such as to furnish rather unsuitable food for the developing of hookworm larvae. Other possible factors will doubtless be found upon further investigation. From the present data it seems probable that the reduction of the numbers of mature hookworm larvae resulting from the ingestion by chickens of hookworm eggs is due to three factors, grinding in the gizzard, differences in osmotic pressure caused by urine in the excrement, and malnutrition of the larvae in the feces.

THE ESTABLISHMENT OF INFECTIVE SPOTS BY CHICKENS.

While the number of infective hookworm larvae which could develop in a chicken's feces in one day is comparatively small, if this number were added day after day at the same place a dangerous infective spot would develop. Such a condition might arise around a drinking fountain or other wet place, where chickens are accustomed to come many times daily. To test this point, several samples of soil in the large chicken pen were again examined for hookworm larvae and found to be negative. The chickens were then given two feedings of Beggar No. 2's stool in the usual way, and the drinking fountain placed at one side of the pen on a spot also moistened by the showers. On the following day, several separate fecal deposits were seen near the drinking fountain, partially crushed into the moist soil. The moist condition continued and in two weeks a half-pint of soil taken beside the drinking dish yielded, through the isolation apparatus, seven infective hookworm larvae.

This finding was verified by testing a sheltered, dry spot several feet away, finding it free from hookworm larvae, moving the drinking fountain to it and making feedings as above. The next day a few fecal deposits were noted near the dish. The ground here was moistened by the splashing of chickens and an occasional wetting to imitate the rain. In a week, a half-pint of soil from this new place gave, through the isolation apparatus, nine infective hookworm larvae.

These positive tests suggested the possibility of an infective spot developing under the roost. Accordingly, a corner of the pen was tested for hookworm larvae and found not to contain them. The roosting frame was then moved into the corner and feedings of the hookworm eggs were made on July 6 and 18. As this corner was sheltered, the accumulating feces were moistened every few days. This mass was largely feces, but a small amount of dirt was scratched into it by the chickens. Two weeks after the last feeding, a pint of the mass was placed in the isolation apparatus, with the result that 157 infective hookworm larvae were counted, 107 being sheathed and 50 unsheathed. These experiments show that chickens swallowing human excrement containing hookworm eggs are able to establish infective spots on moist ground. In the rainy season in Trinidad such spots are likely to develop in any place which regularly attracts the chicken, such as the drinking fountain, the feeding ground (if they be fed) and the roosting place.

Can newly hatched larvae pass uninjured through the digestive tract of chickens?

As hookworm eggs in warm, moist conditions are known to hatch in from 24 to 36 hours, and as chickens may thus swallow young unsheathed larvae, it is important to learn whether such larvae can pass through the digestive tract of the chicken uninjured. A portion of a four-day culture of human feces and animal charcoal containing many unsheathed hookworm larvae was administered to the chickens, when they had not been given feces for several days previously. Three hours after feeding, some of the freshly defecated black feces were examined by smear and five young unsheathed larvae were found in a small portion of it. The feces passed overnight were collected the following morning and thirteen smears taken at random were examined with the result that nine were positive, containing extremes of one and ten active hookworm larvae, making a total of twenty-four. This shows that the unsheathed hookworm larvae can retain their viability while passing through the digestive tract of the chickens. Unfortunately, no test was made to ascertain whether such larvae were able to develop to the infective stage. That they came through active and apparently normal makes it probable that they could reach the infective stage. This hypothesis is strengthened by the fact that newly hatched larvae of another species (*Strongyloides*, cited elsewhere), ingested by the chickens, were able subsequently to develop to the infective stage.

INGESTION OF INFECTIVE HOOKWORM LARVAE BY CHICKENS.

As chickens, in feeding about the yards and edges of the fields, are likely to swallow material containing infective hookworm larvae and later deposit it about the door yard, it seemed important to ascertain whether such larvae could be transported in this way. Four feedings of sheathed hookworm larvae were made, beginning on May 28, when 548 larvae were administered in filter paper and animal charcoal. All of the feces voided during the next 24 hours were collected, but examinations by smears and sedimentation failed to reveal an infective larva. A similar test was made on June 3d, when 500 infective larvae were administered in the same way. Examinations of the feces collected for 21 hours afterwards failed to yield a single hookworm larva.

A third feeding on June 9th was administered with a long pipette, the fluid which contained about 3,500 infective hookworm larvae being darkened with animal charcoal. All of the feces passed by these fowls during the next 22 hours were broken up and placed in a small isolation apparatus. Not a single hookworm larva appeared, whereupon the feces were sedimented, the residue being examined in Syracuse watch glasses and Petri dishes. Still no larvae could be found. The fourth feeding, including 3,400 infective larvae, was made four days later by the aid of a long pipette. In other respects the test was similar to the third just described. When the feces were placed in the small isolation apparatus, five mature hookworm larvae which had lost their sheaths were recovered. Further examination, by sedimentation and centrifugation, failed to yield any more larvae. These tests show that when infective hookworm larvae are swallowed by chickens they do not pass out at once unaltered. They may be digested or they may penetrate the wall of the alimentary canal. That all are digested is highly improbable, since young unsheathed larvae are known to survive passage through the chicken's food tube. Hence, it may be assumed that some of them enter the wall of the digestive tract. How long they remain in the chicken's body is problematical. Any that subsequently leave its body alive have probably so metamorphosed as to be unable to actively enter the body of a final host. That they do not continue development in the intestine of the chicken was determined when the two chickens used in these experiments were examined 67 days after the first feeding. A careful search on the walls of the intestines and through the entire intestinal contents failed to reveal a single hookworm larva. The results of these feedings show that the chicken rarely disseminates infective hookworm larvae. In fact they indicate that in swallowing such larvae the chicken may be a positive factor in hookworm control.

Practical significance.

The results of the various experiments show that in hookworm control the chicken is both beneficial and harmful. From the series of quantitative tests (Table II) it is evident that the great majority of the hookworm eggs swallowed by chickens fail to produce infective hookworm larvae. This is significant, for during the long rainy season in Trinidad a large percentage of the eggs in human stools, deposited out of doors, produce infective hookworm larvae, even though the stools be exposed to the direct rays of the sun during four or five hours daily. (Data on this point will be given by the writer, in this Journal, in a later article of the series.) In such stools thousands of infective hookworm larvae develop in five days, whereas of the portions swallowed by chickens, only a small percentage of the eggs produce mature hookworm larvae. The scarcity of cereals and other ordinary chicken feeds on this island makes it imperative for the fowls largely to take what they can get, so that stools deposited where there are chickens usually disappear in a few hours. In this respect chickens are undoubtedly important positive factors in the control of hookworm disease during the rainy season.

On the other hand, hookworm eggs in stools, deposited out of doors during the dry season, probably all perish when left exposed, but of those eggs swallowed by chickens and dropped at the drinking place, a small percentage would produce infective hookworm larvae. Further, eggs in the fecal particles adhering to the feet of the chickens may also be brought to the drinking place where there is sufficient moisture to protect the developing hookworm larvae. Another detrimental phase of the chicken relationship that may occur either in the wet or dry season is the bringing of hookworm eggs by chickens from somewhat remote places up to the drinking or feeding spot. These spots, usually near the house, are repeatedly traversed by members of the family, most if not all of whom go barefooted.

Taking into consideration all of the *pros* and *cons* in the case, the writer is of the opinion that the great reduction of infective hookworm larvae resulting from the ingestion of quantities of human excrement by chickens outweighs the harm done by them in carrying eggs in their digestive tracts or on their feet to the door yard where infective spots may be established. In other words, the chicken does more good than harm. But in unsanitated districts or places where only certain members of the family use the latrine, the chickens are able to establish infective spots which in the door yard would be constant sources of danger.

STRONGYLOIDES STERCORALIS IN CHICKEN FECES.

Besides hookworm eggs in the stools used in the experiments, the eggs of *Ascaris lumbricoides* and *Trichuris trichiura* were of occasional occurrence, and a few newly hatched larvae of *Strongyloides stercoralis* were seen in the smears. These young larvae were able to survive passage through the chicken's body. No special studies were made to determine the minimum number of days required for them to develop to the infective stage in a culture medium of chicken feces and animal charcoal, or soil, but by 7 days after their passage through the chickens, fairly large numbers of them had developed the characteristic long filariform oesophagus, and were rapidly wiggling their way through the cultures. A single active mature male of the free-living generation was seen in a ten-day-old culture.

SUMMARY.

1. The length of time required for food material to pass through the digestive tract of chickens ranges from two hours and forty minutes to sixteen or more hours.

2. Hookworm eggs remain viable while passing through the alimentary canal of chickens and are able to hatch.

3. Hookworm eggs swallowed by chickens produce infective hookworm larvae when the fowl feces are mixed with animal charcoal or with soil.

4. The results of a series of experimental feedings indicate that

the great majority of hookworm eggs ingested by chickens fail to produce infective hookworm larvae. This failure is attributed in part to breaking of eggs in the gizzard, injury from urine in the feces, and to malnutrition of the larvae in the excrement.

5. Dangerous infective spots may be established around drinking receptacles by chickens that have swallowed hookworm eggs day after day.

6. Hookworm eggs deposited out of doors under conditions unfavorable for development can be carried by chickens to favorable environments.

7. Human stools voided in unfrequented places can be transported by chickens to the door yard which is traversed by bare-footed persons.

8. Newly hatched hookworm larvae can pass through the digestive tracts of chickens apparently uninjured.

9. Sheathed hookworm larvae swallowed by chickens fail to pass at once through the fowls unaltered.

10. *Strongyloides stercoralis* larvae occurring in the human stools passed through the chickens and soon attained the infective stage, a single mature male of the free-living generation having been seen.

11. The various experiments first performed on adult chickens were later repeated with young ones with similar results in each case.

12. A comparison of the reduction of mature hookworm larvae by fowls with the establishment of infective spots by them convinces the writer that chickens are more beneficial than harmful in the control of hookworm disease in Trinidad.

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INVESTIGATIONS ON THE CONTROL OF HOOKWORM DISEASE.

V. THE DOMESTIC PIG AND HOOKWORM DISSEMINATION.*

By JAMES E. ACKERT, Ph.D., and FLORENCE KING PAYNE, M.D.

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INTRODUCTION.

The free range frequently allotted the domestic pig, and its omnivorous habit have led to the suspicion that swine may be factors in hookworm dissemination. Experiments conducted by the Philippine Bureau of Science in 1913 indicated that the domestic pig could not be infected with human hookworms. Whether or not pigs may act as vectors in the dissemination of human hookworm eggs was not considered by them. In the absence of any record of experiments, and in order to investigate this point, the writers took advantage of an opportunity afforded in Trinidad, British West Indies, by the International Health Board of the Rockefeller Foundation, through the efforts of Dr. W. W. Cort and Dr. George C. Payne, to whom acknowledgment is here made for valuable suggestions.

METHODS.

For the experiments three pens were used, a living pen, an experimental feeding pen and a small enclosure used only for collecting feces from experimental feedings. These pens, which were sheltered by the laboratory (Ackert, 1922), were free from hookworm larvae.

Two young pigs, approximately six weeks old, were used for the feeding experiments. Examinations of their feces by smears, sedimentation and centrifugation, and by the brine-loop-flotation technique failed to reveal the presence of any nematode eggs or larvae. By administering animal charcoal with food it was ascertained that sixteen hours were required for material to pass through the pigs. The human stools, which were mixed with corn meal and milk, and sometimes animal charcoal, were administered in the experimental feeding pen, care being exercised to avoid polluting the soil. The pigs were then placed in the small recovery room and the feces collected during the following twenty to twenty-fours hours. Upon recovery their feces containing hookworm eggs were cultured in animal charcoal or in garden soil which had been sufficiently heated to kill any hookworm eggs or larvae, but not hot enough to oxidize organic material. After a week or so the cultures in garden soil were placed in separate units of the isolation apparatus (Cort et al., 1922) which aided in isolating the infective larvae.

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* This paper is the fifth of the series of the hookworm investigations made by the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University with the Cooperation of the International Health Board, Rockefeller Foundation. It is Contribution No. 56 from the Department of Zoology, Agricultural Experiment Station, Kansas State Agricultural College.

Can eggs swallowed by pigs produce infective larvae?

In the tropics and sub-tropics the ingestion of quantities of hookworm eggs by pigs is a daily occurrence. Countless numbers of these eggs are dropped in environments which doubtless enable them to hatch. But as the young, unsheathed hookworm larvae are known to be unable to penetrate the skin of their final host, the feces of the pig must furnish sufficient nutriment to enable the hookworm larvae to reach the infective stage, if the pig is a real factor in the spread of hookworm disease.

To test this point two cubic inches of human stool from Beggar No. 1 (light infestation), containing many hookworm eggs, were administered to the pigs. The feces passed during the following 24 hours were cultured in animal charcoal and placed in Petri dishes. In six to eighteen days afterward 72 active infective hookworm larvae were isolated from these cultures. This experiment was repeated by giving to the pigs 3 cubic inches of human stool. The feces collected after 16 hours were cultured in animal charcoal and placed in a large moist chamber. Examinations of this culture by washing from the 10th to the 17th day afterward yielded 289 infective hookworm larvae, apparently similar in every respect to hookworm larvae from human stools cultured directly in animal charcoal. These and many subsequent tests showed conclusively that hookworm eggs swallowed by pigs are able to produce larvae which develop to the infective stage in a culture medium of pig feces and animal charcoal.

As animal charcoal is known to inhibit putrefaction it appeared that the chances of young hookworm larvae reaching the sheathed stage would be less favorable in a medium lacking the charcoal. To settle this point, one and a half cubic inches of stool from Beggar No. 2 (heavy infestation) were administered to the pigs without any charcoal. The resulting feces were mixed with previously heated garden soil and placed in an open earthen pan. Examination by the aid of the isolation apparatus nine days later yielded 871 vigorous infective hookworm larvae. This demonstrated that hookworm eggs which had passed through the digestive tract of the pig produced infective larvae in the absence of animal charcoal.

WHAT PROPORTION OF HOOKWORM EGGS SWALLOWED BY PIGS REACH THE INFECTIVE STAGE?

Having determined that hookworm eggs swallowed by pigs produce infective larvae under approximately natural conditions, an attempt was made to obtain a comparison of the number of sheathed hookworm larvae developing from equal portions of a stool when one half of it was cultured directly and the remainder given to a pig. For this series of tests Pig. No. 1 was chosen because of its voracious appetite. The essential data in these tests are shown in Table I.

TABLE I.

Comparison of culturing one half of stool directly with giving other half to pigs.

Cubic inches of stool.	Disposal of stool.	Feces recovered hours after feeding.	Culture medium.	Environment.	Days cultured.	Infective larvae obtained.
4..........	Half cultured		Soil	Shade	9	1,356
	Half to pig	21	Soil	Shade	8	3,003
3..........	Half cultured		Soil	Sun and shade	8	103
	Half to pig	24	Soil	Sun and shade	8	1,160
2..........	Half cultured		Soil	Sun and shade	11	18
	Half to pig	44	Soil	Sun and shade	10	897
3..........	Half cultured		Soil	Shade	8	957
	Half to pig	24	Soil	Shade	7	1,720
4..........	Half cultured		Soil	Shade	7	3,672
	Half to pig	25	Soil	Shade	6	2,520

For this series of feedings the stool of one individual, known as Beggar No. 2, was utilized on account of the large number of hookworm eggs per volume in it, and because of the relative freedom from eggs and larvae of other nematodes. The " half " stools are approximate, having been divided optically. The culture medium in each case was previously heated clay loam, and the culture dishes, eleven-inch tin pans (two inches deep), perforated for drainage. The " shade " environment was either deep shade out of doors or in a small yard house. In the " sun and shade " environment the cultures were placed out of doors, where they were exposed to approximately four hours daily of direct sun rays and to the tropical showers.

In the first test the feces were scratched into the surface of the soil and kept moist by sprinkling, except on two days when they were placed out in heavy showers. The second test was practically a duplication of nature, the human stool and pig feces being placed on top of the soil and set out of doors side by side. There were short heavy rains nearly every day and much sunshine. The relatively small number (103) of larvae, obtained from the human portion, was probably due in part to the rapid disappearance of the stool, no visible trace of which was left at the end of the third day. This was due to the combined action of fly larvae and the rain, and to the nonfibrous condition of the stool. If adverse climatic conditions prevailed, just as the hookworm eggs hatched, the exposed larvae in such a culture would probably perish before they could migrate to shelter. On the other hand, the fibrous pig feces, which were spread out by the rain, formed a disk-like covering over the surface of the culture, affording protection for the young larvae against the intense rays of the sun and the force of the heavy rains. The conditions of the third test were similar to those of the second one, the soft human stool disappearing during the third day, when most of the larvae are in a precarious condition.

That the rays of the sun and the beating rains are especially unfavorable to hookworm larvae in soft human stools is indicated by the results of the fourth and fifth tests. The conditions of these tests were similar to those of the second and third tests, except the meteorological ones of sun and rain. These cultures, kept in deep shade in the yard house and gently sprinkled, yielded many more larvae. The fly larvae appeared to be almost, though not quite, as numerous as they were out of doors. Just what effect the fly larvae have on the developing hookworm larvae is a problem in itself. They undoubtedly aid in making the stool porous, admitting oxygen more quickly, but they usually break the protective crust, and as they feed wholly on the stool, the amount of nutriment is certainly reduced. Since nutriment is an important factor in the development of hookworm larvae, a reduction in number in such fly-infested stools would be the natural course of events.

The majority of the fly larvae in the human stools were those of *Drosophila*, apparently attracted by the undigested pieces of banana and other fruits. A smaller number of flies were drawn to the pig feces which contained many oat hulls and plant fibers. These not only formed a protective crust, but probably kept the food particles more concentrated for the developing larvae. At any rate, these tests indicate that under the present conditions in Trinidad, hookworm eggs, swallowed by pigs, have fully as favorable prospects for producing infective hookworm larvae as do those that remain in the human stools deposited out of doors.

Uncontrolled factors and possible errors in technique of course may enter into these results. The optical division of the stools into "halves" is approximate rather than accurate, but differences in volume of four or five per cent. are readily detected. The distribution of the eggs in the stools may vary, but in each one of several different microscopic examinations of Beggar No. 2's stool, hookworm eggs were found in the first smear. A possible error in counting must of course always be considered, but the numbers of infective larvae obtained from the pig cultures were so large that a high percentage of the hookworm eggs swallowed by pigs are evidently able to produce sheathed larvae during the rainy season in the tropics.

FATE OF INFECTIVE HOOKWORM LARVAE SWALLOWED BY PIGS.

As pigs in feeding and drinking about the yards are likely to swallow human hookworm larvae in the infective stage it was deemed advisable to ascertain if such larvae might be disseminated by the pigs. This question arose early in the work, and on June 16, 2,841 active infective larvae were administered to the two pigs in milk, in a soup plate, care being exercised to have the larvae swallowed. The feces passed during the next 24 hours were separated and placed in a unit of the isolation apparatus. Many free-living nematodes, but not a hookworm larva could be obtained in this way. The feces were then broken up and prepared for sedimentation. A further search likewise proved futile, not a single hookworm larva being found.

Four days after the first feeding, a second test was made by giving to one of the pigs 2,700 infective hookworm larvae in milk, the latter being stirred while the pig was drinking it. A second volume of milk added was lapped up clean, leaving no doubt that most, if not all, of the larvae were swallowed. The feces voided by this pig during the next 22 hours were teased and placed in the isolation apparatus, but in this test, as in the first, not a single hookworm larva could be obtained. As a check on the isolation apparatus, 72 sheathed hookworm larvae were placed in some pig feces that were free from larvae. This excrement was placed in the isolation apparatus under conditions similar to those of the second test with the result that 26 sheathed hookworm larvae were recovered.

The third and final feeding of infective hookworm larvae was made on June 27, when 3,000 sheathed larvae were given to one of the pigs in milk as before. To guard against the possibility of the larvae escaping from the feces, the latter were made to fall on clean dry paper. Feces from this pig were collected during the following 25 hours, the last fecal deposit having been taken immediately after defecation. This excrement was teased and placed in the isolation apparatus, with the final result that no hookworm larvae could be found. The fate of such ingested larvae is, at present, unknown. It is probable that they either penetrate the wall of the digestive tract or die in the attempt, in which case they would be more or less completely digested. To determine the fate of those which might enter the wall of the alimentary canal further investigation is necessary.

STRONGYLOIDES STERCORALIS IN PIG EXCREMENT.

In addition to the abundance of hookworm eggs occurring in the human stools used in these experiments there were a few eggs of *Ascaris lumbricoides* and *Trichuris trichiura,* and an occasional larva of *Strongyloides stercoralis.* It was soon very apparent that the *Strongyloides* larvae could survive passage through the digestive tract of the pig, for in the cultures of pig feces these larvae in the free-living generation had multiplied to the extent of being numerous.

Among the results obtained by the Philippine Bureau of Science in 1913 from feeding human waste to pigs were indications that the pig may become a host of *Strongyloides stercoralis.* With numerous infective larvae of this species available from time to time in the present experiments it seemed desirable to attempt to collect more data on this problem. As stated elsewhere the feces of the two six-week-old pigs secured for the hookworm experiments were free from eggs and larvae on June 11. Many infective *Strongyloides* larvae were administered along with the infective hookworm larvae on June 16, 20 and 27. But on June 24 approximately 3,000 infective *Stron-*

gyloides larvae were given to one of the pigs. No visible effects of this dose were noted until three days later, when the pig refused to eat and vomited twice, remaining quiet the remainder of the day. By the next morning, however, the pig appeared normal again, and no further possible effects could be detected.

As time elapsed, the numbers of *Strongyloides* larvae in the pig feces cultures increased, indicating that the pigs were contributing some of the larvae. To test this point, the pigs were placed on a woven wire floor in the small feces recovery pen, where they were given food free from *Strongyloides* larvae. After allowing 28 hours for any ingested *Strongyloides* to pass from the pigs, the space beneath the wire floor was cleaned and covered by a clean paper. The pig feces were then collected for 18 hours and the major portion of them cultured in previously heated soil, in the usual way, on August 5. In 8 days the culture was examined by the aid of the isolation apparatus. After centrifugation the centrifuge tube contained a solid mass of these larvae, approximately three centimeters deep. By diluting and stirring this mass of larvae, counting those in a single drop, and multiplying by the number of drops, the total number obtained amounted to 68,400 *Strongyloides* larvae.

As a check one and a half cubic inches of Beggar No. 2's stool was cultured in previously heated soil and placed near the pig feces culture. Seven days later examination of this culture by aid of the isolation apparatus yielded approximately 200 *Strongyloides* larvae. The contrast here is so great that there appears to be no other interpretation than that these pigs were infested with mature *Strongyloides* of the parasitic generation. The 42 days which elapsed after the first feeding of infective larvae doubtless afforded ample time for development to maturity in the pigs. While these tests do not preclude the possibility that the pigs became infested with a species of *Strongyloides* other than *stercoralis,* the care and feed of the pigs was such as to make it highly probable that it was the human species.

An interesting observation on the behavior of the free-living generation was made. On looking at the surface of one of the pig feces cultures some white wavy specks were noted. With the aid of a hand lens it was seen that these specks consisted of groups of *Strongyloides* larvae in the infective stage, the individuals being wound around one another, much like the strands of a thread, except that at the extreme distal end of the group a portion of one larva extended beyond the others. This and dozens of other masses were waving to and fro on the surface of the moist culture, certainly in favorable positions should the host come in contact with the surface of the culture.

HOOKWORMS IN PIGS.

Late in the season, an additional feeding of hookworm eggs was made in the usual way, one half of Beggar No. 2's stool being cultured directly and the other half administered to the pigs. The feces from this feeding were cultured and placed in the shade beside the human culture. Examinations of these cultures eight days later showed 1.200 infective larvae from the human culture and 6.203 from the pig culture. This latter number was so large—twice the number from any previous pig culture—as to lead to the suspicion that the pigs might be contributing hookworm eggs. Accordingly, a test was made in which the pigs were given carefully selected food for a day, after which their feces were collected and cultured. In a week, the examination by the aid of the isolation apparatus yielded 4.294 infective hookworm larvae. These results tended to confirm the suspicion that the pigs had become infested with hookworms, and led to an immediate examination of the pigs' intestines.

In the intestine of the first pig slaughtered (Pig No. 2) five small blood clots were found in the ileum, and near them eight mature hookworms, five females and three males. The results from the examination of the second pig (No. 1) were similar, except that three mature hookworms were found, two females and one male. To ascertain whether hookworms were occurring in pigs kept under ordinary conditions, a three-month-old pig was purchased and slaughtered. From the posterior third of its small intestine 137 hookworms were taken, six pairs being in the act of copulation. Further examinations of pig intestines from various parts of the island showed that the infestations were not limited to young pigs, nor to particular geographical areas. Data on these points are still being assembled, and will be published in a later article of this series.

Studies on the morphology of the hookworms from several of these pigs convince the writers that these nematodes belong to the genus *Necator*, but differ from *Necator americanus* and other species of this genus to such an extent as to constitute a new species. A diagnosis of the new species is here given, but a full description of it together with data on its occurrence and distribution in Trinidad will be published as a later number of this series of papers.

THE GENUS NECATOR, STILES, 1903.

Hookworms belonging to this genus have a small mouth capsule, narrowed anteriorly (ventrally) by chitinous plates. On each side of the base of the dorsal cone is a lateral chitinous plate or lancet with a smooth edge (not serrated); ventral lancets present as in *Ancylostoma*. No ridges occurring on outside of ventral wall. Opening of the dorsal oesophageal gland on tip of a cone (dorsal tooth) projecting freely into the buccal capsule. Bursa closed. Dorsal ray of bursa divided to its base.

Necator suillus n. sp.

Specific diagnosis.—With the characters of the genus. Inner edges of ventral plates rounded. Dorsal tooth very short. Lateral lancets broadly wedge-shaped in profile. Ventral lancets rather slender; dagger-shaped in side view; point toward base of dorsal tooth. Oesophagus comparatively short and slender.

Male 5.25 to 8.75 mm. long by .09 to .246 mm. in thickness; average length, 6.9 mm. Buccal capsule 57 to 75 microns long (dorso-ventrally), average 65; by 58 to 77 microns wide, average 68. Bursa funnel shaped. Dorsal rays short and comparatively stout: terminal branches of dorsal rays long and slender; externodorsal ray short and stout; posterolateral, mediolateral and externolateral rays short and slender; lateroventral and ventroventral rays stout. Spicules .423 to .471 mm. long and barbed at extremity.

Female 7.3 to 12.5 mm. long by .295 to .382 mm. in breadth; average length, 9.5 mm. Buccal capsule 68 to 89 microns long, average 74; by 67 to 94 microns wide, average 75. Vulva near equator of body, but anterior to middle. Eggs 56 to 66 microns long by 35 to 40 microns wide, in early segmentation when deposited; average length, 63 microns; average width, 37 microns.

Among the morphological differences between *Necator americanus* and *N. suillus* are the following: The new species is somewhat smaller; the buccal capsule much smaller proportionately. As a rule, the dorsal turn in the neck is not so pronounced as in *N. americanus*. The inner edges of the ventral plates are more rounded in *N. suillus*, and less angular than those of *N. americanus*. In *N. suillus* the lateral lancets are broadly wedge-shaped in profile, while those of the larger *Necator* are cusp-shaped. The ventral lancet is slender in side view, pointing toward the base of the dorsal tooth in *N. suillus*, while this lancet in the larger species is broader and points approximately toward the tip of the dorsal tooth. The dorsal rays in the new species are shorter, while their terminal branches are actually longer than these structures in the larger *N. americanus.*

Discussion.

The finding of mature male and female hookworms in pig No. 1 stimulates a re-examination of the data shown in Table I, and of the results from the preliminary feeding experiments. Among the questions which arise is that of the time at which these hookworms probably matured. As they are of the smaller species, *N. suillus*, they probably had entered the bodies of the pigs before June 11, when we secured them and found that their feces were free from hookworm eggs. The preliminary feeding experiments described above were begun at once, and the comparatively large numbers of infective hookworm larvae obtained from the pig cultures stood out in marked contrast to the small numbers obtained from similar chicken cultures (Ackert, 1922). The 871 vigorous infective hookworm larvae from preliminary pig feedings were obtained from feces passed four days after the pigs were secured. It will be seen that the one and a half cubic inches of Beggar No. 2's stool administered to the pigs in this case produced about as many infective hookworm larvae as developed in the average human cultures shown in Table I. This would indicate that the worms had not matured when this feeding was made. and it would also indicate that a high percentage of the human hookworm eggs swallowed by pigs are able to produce infective larvae during the wet season in Trinidad.

The tests for the data of Table I were begun June 19 when the pigs were about seven weeks old. If *N. suillus* matures as quickly as *N. americanus,* it is possible that the three hookworms found in pig No. 1 had matured at this time. On the other hand, they may not have matured until late in the season—the last of July—when 6,203 infective hookworm larvae were produced in a single pig culture, as compared with 1,200 from the corresponding human culture. However, as Calmette and Breton (1905) have shown that human hookworm eggs pass through the digestive tract of the dog unharmed by the stomach, intestinal, and pancreatic juices, there appears to be no reason for doubting the validity of the preliminary tests in the present experiments. These tests indicate that not only do human hookworm eggs pass uninjured through the alimentary canal of the domestic pig, but the majority of them produce infective larvae in a culture medium of pig feces and moist soil.

Anchylostoma in Swine.

After the preparation of this manuscript some references on *Anchylostoma* in swine came to our attention. O'Connor (1920) working in Funafuti, Ellice Islands, found thirty specimens of *Anchylostoma* in a pig. According to this author, the worms were very similar to *A. duodenale,* except for size, being smaller than this human hookworm. In July, 1921, Maplestone reported on the examination of 157 slaughtered pigs and on 25 fecal examinations of swine in North Queensland. No hookworms or eggs were found in any case. As only "the duodenum and first part of the small intestine, with its contents," were examined, any hookworms in the ileum and posterior portion of the jejunum of the slaughtered pigs would not have been found. In Trinidad, the present writers found *Necator suillus* more frequently in the ileum and jejunum than in the duodenum. Apparently, O'Connor found some *Anchylostoma* in the ileum and jejunum. More recently, Legg and Rheuben (1921) have reported the finding of hookworms in three semi-domesticated pigs from North Queensland, stating that the nematodes obtained had been identified as *Anchylostoma duodenale.*

SUMMARY.

1. Human hookworm eggs swallowed by domestic pigs produce infective larvae in five days during the rainy season in Trinidad.
2. The results of tests indicate that a high percentage of the hookworm eggs ingested by pigs are able to produce infective larvae, and that the free-range pig is an important factor in the dissemination of human hookworm eggs.
3. Infective hookworm larvae swallowed by pigs do not pass through unaltered in the excrement.
4. *Strongyloides stercoralis* larvae survive passage through the digestive tract of the pig and multiply in the feces. Circumstantial evidence shows that the pigs became infested with this nematode.
5. A new species of hookworm, *Necator suillus*, is of common occurrence in the domestic pigs in Trinidad, British West Indies.

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THE RELATION BETWEEN FATIGUE AND THE SUSCEPTIBILITY OF RATS TOWARDS A TOXIN AND AN INFECTION.*

By ELLA HUTZLER OPPENHEIMER and REYNOLD A. SPAETH.

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PRELIMINARY.

There is a popular belief in the medical world today that a fatigued individual is more susceptible to disease than a non-fatigued individual. There are no available statistics to prove or disprove this opinion. All that is known is that workers in industry, and they necessarily must be included in the group of " fatigued individuals," become infected as do the idle onlookers. There are no comparative figures on the susceptibility rates for the two groups.

This series of experiments attempts to discover whether a state of fatigue will produce in experimental animals a greater susceptibility to toxins and infections. By the term " fatigue " we mean a condition of temporary exhaustion produced by excessive muscular exertion. In our work with tetanus toxin, we found that while fatigue did not markedly reduce susceptibility, neither did it increase it in white and hooded rats. There was, however, a definite tendency for fatigue to increase the resistance of these rats towards an infection of pneumococci.

PREVIOUS WORK ON FATIGUE AND SUSCEPTIBILITY.

Although we found no record of any work on the relation between fatigue and susceptibility to toxins, we discovered in the literature accounts of similar work by two groups of investigators on the influence of fatigue on susceptibility to infections. Their results were not the same.

In 1890, Charrin and Roger (1) (2) injected 22 white rats with anthrax bacilli of different virulence. Of the 11 rats injected with " charbon bacteridien," 3 unfatigued controls all survived. The other 8 rats were fatigued after injection. One of these survived; 2 died within 24 hours; the other 5 died after 3 days. Eleven rats were injected with "charbon symptomatique." The 5 controls all lived. The 6 rats fatigued after injection all died; 3 within 24 hours, 2 others after 30 hours and 1 after 50 hours. These results indicate that fatigue after the injection of anthrax bacilli favors the development of this infection in the white rat.

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* From the Physiological Laboratory, School of Hygiene and Public Health, Johns Hopkins University.

In 1910, Abbot and Gildersleeve (3) fatigued 18 rabbits and found that there was always a reduction of the opsonic index. From this it was expected that the susceptibility to the pyogenic organisms would be heightened, but the results were indefinite. Of 7 fatigued rabbits injected intravenously with *Streptococcus pyogenes,* 6 died of fatal septicemia from 6 to 30 days earlier than the controls. The seventh rabbit died at the same time as the control. The results with *Staphylococcus pyogenes* were entirely negative. Eleven exhausted animals with lessened opsonizing powers did not react differently from the controls when inoculated intravenously or subcutaneously.

These results, in so far as they are positive, confirm the generally accepted conception of the relation between fatigue and "natural susceptibility."

EXPERIMENTAL.

The animals with which we worked were rats, white and hooded. In spite of the fact that the M.L.D. determinations for both tetanus and diphtheria toxins had been made for guinea pigs, we substituted the rats because of the ease with which they run and can be fatigued. The size of the rats was an additional advantage.

All of our rats were vigorous, healthy animals and throughout the period of experimentation no abnormalities of any kind appeared in the stock. None succumbed to extraneous infections. The rats were fed once daily a uniform McCollum diet of the following formula:

3 parts oat meal
3 parts whole wheat flour
3 parts corn meal
1 part flax-seed meal.

PRELIMINARY EXPERIMENTS WITH TETANUS TOXIN.

In the first experiment we tried to discover whether fatigued rats are more susceptible to tetanus toxin than normal, healthy rats of the approximate age and size and under the same surrounding conditions of temperature, humidity and light. Before this could be determined the M.L.D. of tetanus toxin had to be established for white and hooded rats.*

From information obtained from the literature and other sources, we were led to believe that rats are highly resistant to all toxins including tetanus toxin. But as the rats are such ideal animals for exercising and fatiguing, we attempted the M.L.D. determinations for the tetanus toxin even though it appeared to be unfavorable. Therefore, as the appended table shows, we began by giving the rats large doses of the toxin and decreased the doses gradually. The M.L.D.—that is, the dose of toxin that would kill a rat within a week—was 0.00,000,004 gr. toxin per gram weight of rat. For a 150 gr. rat, therefore. the dose was 0.00,000,6 gr. toxin. The lethal dose, however, is much less, if no time limit be placed upon death. In our experiments we used a just sublethal dose and although this did not kill, it usually paralyzed the rat. In some cases we used a just lethal dose so that the effect of the fatigue, if there were any, could be more plainly seen.

Table I in the Appendix shows the effect of the tetanus toxin in the determination of its M.L.D. On the basis of these determinations, we decided to use doses of 0.00.000,002,6 and 0.00.000,002,4 gr. tetanus toxin per gram weight of rat for the fatigue experiments. The toxin after being weighed was dissolved in normal saline solution and injected subcutaneously.

APPARATUS.

The apparatus used for fatiguing the rats was essentially similar to that of Richter (4). It consisted of two sets of wire drums, three drums in each set. The drums were made of one quarter inch mesh galvanized wire net. The dimensions and details of a single drum are shown in Fig. 1. Each drum was mounted on a bicycle hub. This we found necessary since, when an axle ran through the center of the drum, the rats would perch upon this axle instead of running. The total distance run was recorded on each drum separately by a Veeder cyclometer arranged with a trip lever as shown in the figure. At the front of each drum, there was a heavy drop door lined with zinc and held in place by a spring catch. The circular one half inch oak back

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* We are indebted to Dr. George W. McCoy for the toxin used in these experiments, and to Dr. J. P. Leake for the method of handling the toxin.

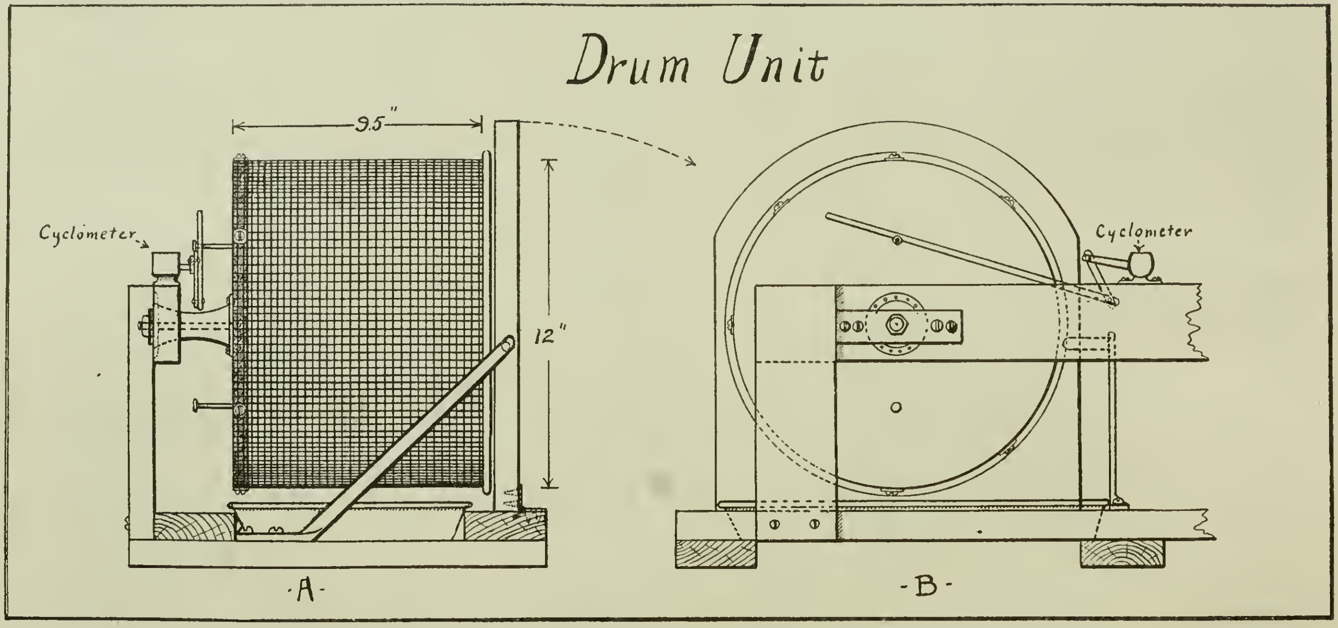

Fig. 1. *A*, side, and *B*, rear view, of a drum unit used in fatiguing the rats. Six drums in two sets of three each were used in the experiments. Heavy canvas webbing, one and a quarter inches wide, was used as belting. The drums themselves acted as pulleys. The frame was made of one inch redwood.

of each drum was likewise lined with zinc. The complete set-up consisted of six drums belted in parallel, two sets of reducing pulleys, a motor, and rheostat.

METHOD OF FATIGUING ANIMALS.

We attempted at first to make the rats spontaneously fatigue themselves in these drums as in a treadmill. But while the moving drum is actually a stimulus which serves to keep the rats running, we could not count on an end result of fatigue. Following Richter (loc. cit.), rats were run for a series of days, 6 hours at a time with 6 hours of rest intervening. At first their activity was slight but it gradually increased, following a regular learning curve. After a few trials, they would run as much as 4 miles in the 6-hour interval, but before there was any evidence of fatigue, they would spontaneously rest. Thus, the rats, instead of becoming exhausted by fatigue, improved their physical condition by this form of exercise.

In fatiguing the rats, therefore, the drums were turned by an electric motor. Under these conditions spontaneous rest was practically eliminated. By a series of pulleys and a rheostat, we could regulate the speed so that a definite distance would be covered in a given interval. When the rats fought against the revolving drum, they were quickly fatigued. Exhaustion came less rapidly when the drum was turned at a slow rate for a long time than when it revolved rapidly for a short time. In most cases as fatigue became pronounced, the rats barely moved one foot after another and as soon as the drum ceased moving, they immediately fell asleep.

MEASUREMENT OF FATIGUE.

The entire distance covered by the rats could be computed by multiplying the circumference of the drum by the number of revolutions. We attempted at first to run each rat for the same time and over the same distance, hoping that the fatigue resulting would be approximately the same. But this was not the case. The greater the ease with which a rat ran, the longer it took to become fatigued.

Therefore, in fatiguing the rats, the result looked for was not fatigue in terms of distance or time run, but in terms of the effect on the rat and its physical state. When it was so greatly fatigued that it no longer ran, was carried about by the drum, and, in general, failed to respond to auditory or tactile stimuli and was content merely to lie and rest, we considered the rat exhausted and completely fatigued (5). Our criteria of fatigue were, therefore, purely empirical and qualitative.*

Although the rats lost weight whenever they reached this stage, the condition was not lasting. This was proved by running them on several succeeding days. After running for 4 hours on one day, they usually had completely recovered their normal weight before running again the next day. Therefore, the rats were not permanently harmed, but were, indeed, only fatigued.

Adjustment of running periods and injections.

At first, we fatigued the animals once immediately before injection. As this seemed to have no effect on the rats' susceptibility, we increased the amount of fatigue by exhausting the rats in three successive periods. They were fatigued two days before, the day before, and immediately preceding the injection. Other rats were injected before being fatigued. In the case of the tetanus toxin, we assumed that unless fatigue occurred after the latent period of the toxin had passed, the effect would be approximately the same as fatigue before injection. Therefore, the rats were fatigued not only immediately after receiving their dose of the toxin, but also on the two succeeding days. In this way, both toxin and fatigue could be effective as the latent period of the former was surely completed.

At least 3 rats were injected at the same time in each experiment. One rat served as a control. It was injected with the same dose as the rat fatigued before injection. A third rat was injected and subsequently fatigued as described above. The general experimental scheme was, therefore, as follows:

Group 1. Rested ....injected........rested (control)
Group 2. Fatigued ......injected........rested
Group 3. Rested ....injected........fatigued

Effects of fatigue on susceptibility to tetanus toxin.

In the case of tetanus toxin the variation in the susceptibility of fatigued and normal rats was slight and was not always in the same direction.

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* In a few cases, the rats discovered that by lying on their backs and raising their feet, the drum would slip by beneath them without seriously disturbing their position. When violent tapping on the cage no longer made these animals jump to their feet and begin running, they were considered exhausted.

Of the 22 rats injected, none of the 8 animals fatigued before injection died before the controls; 1 was affected in the same way as the control, and 2 of these rats were paralyzed more severely than the controls and were slower in recovering. Of the 8 rats fatigued after injection, 1 died before the control; 4 survived the controls; 1 died at the same time; 1 was paralyzed more severely and took longer to recover than did the control, and 1 was paralyzed more severely but recovered at the same time.

Fatigue, therefore, increased the susceptibility of 6 rats (37 per cent.), increased the resistance of 9 rats (56 per cent.), and failed to affect 1 rat (6 per cent.). Although there are too few cases to warrant any very positive conclusions, we were nevertheless struck by the fact that fatigue apparently increased the resistance rather than the susceptibility of the majority of our animals.*

The experimental results will be found in detail in Table II of the Appendix.

Relation between fatigue and pneumococcal infection.

We selected Type I pneumococcus as a suitable organism for testing the relation between fatigue and infection. We used 18-hour broth cultures of a virulent organism and diluted it to the desired strength with sterile broth. We made intraperitoneal injections.†

We continued using white and hooded rats as we found these animals to be most satisfactory. We used the same apparatus as in the tetanus toxin experiments and the animals were fatigued in the same way. That is, we considered an animal exhausted when it could no longer run and rolled on its back and was carried about by the drum—whether it had run 1 hour or 6, or had covered 1 or 4 miles. Some of the rats we fatigued once before injection; some were fatigued three times before injection; some were fatigued once after injection, and others in three periods after having been injected.

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* We desired to use another toxin to discover whether we could get comparable results. We, therefore, attempted to find the M.L.D. of diphtheria toxin for white and hooded rats. In this case, however, so great a dose is necessary to kill a rat that we were unable to determine it. In smaller doses, up to 2 c.c., the toxin affected the rats quite like injections of a similar amount of normal saline solution.

† In the course of the work with pneumococcus, an observation was made that seems worth recording. Eleven rats developed a pathological condition of the eye from the injection of the pneumococci. In two cases, both eyes became completely opaque; the others were affected in only one eye. In only two of the eleven rats did the eyes clear up. The autopsies showed that in no case was the lens cloudy, the opacity being limited to the cornea.

Seventy-eight rats were injected; 18 of these were unfatigued controls and the other 60 rats were fatigued. In this case, a clear-cut result was obtained. Fatigue not only failed to lower the rats' resistance to the pneumococci, but actually decreased their susceptibility to this infection (see Fig. 2).

Of the rats fatigued before injection, 26 survived the controls; 1 died before the control; 1 died simultaneously with the control, and 2 lost weight and recovered with the controls. Of the rats fatigued after being injected, 15 survived the controls; 7 died before the controls; 5 died at the same time as their controls, and 3 lost weight and recovered with their controls. In other words, fatigue increased the resistance of 67 per cent. of the rats, and only increased the susceptibility of 13 per cent. The susceptibility of 20 per cent. was unaffected.

As a concluding experiment in the above series (Series C, Experiment 11), we injected 17 rats—6 we used as controls, 6 we fatigued before injection and 5 we fatigued after injection. Five of the 6 controls died in 3 to 6 days; the 6 rats fatigued before injection all survived and 3 of the others died in 4 to 5 days. That is, 5 out of 6, or 86 per cent. of the control rats died, and only 3 of 11, or 27 per cent. of the fatigued rats died. This experiment definitely confirmed our earlier results showing an increased resistance to pneumococcal infection produced by fatigue. Fig. 2 shows the results of experiments 9, 10 and 11, Series C. In every case the rat received 0.0002 c.c. of a culture that had been transplanted twice. All rats below the zero line lived: all above, indicated by black rectangles, died.

A study of Table III in the Appendix shows that fatigue *before* the injection of pneumococci is nearly twice as potent (86.6 per cent.) in raising the resistance to this infection as is fatigue which follows the pneumococcal injection (50 per cent.). We have no explanation to offer at present either for the general fact of increased resistance resulting from fatigue or for the greater resistance produced by fatigue which precedes the injection. Further experiments, now in progress, may throw some light on a physical-chemical mechanism that will account for the phenomena.

General conclusions.

1. The susceptibility of white and hooded rats to tetanus toxin is far greater than has hitherto been supposed. The M.L.D. is 0.00,000,004 gr. toxin per gram weight of rat.

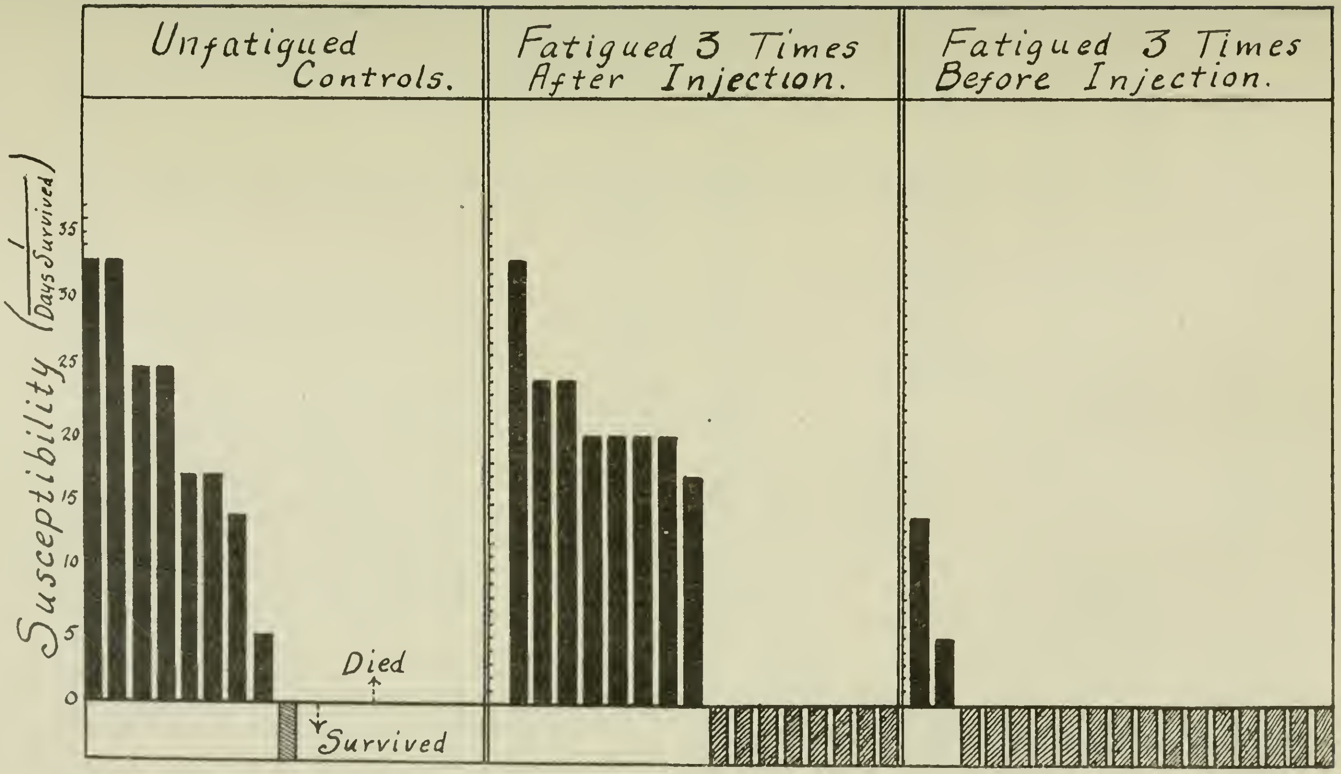

FIG. 2. A composite chart showing the relation between fatigue and susceptibility to pneumococcal infection in the forty-two rats of Experiments 9, 10, and 11, Series C. Each black rectangle represents a single rat that died from an injection of 0.0002 c.c. of our virulent strain of pneumococcus. The cross-hatched rectangles below the zero line represent rats that survived the experiment; i.e., rats that lived indefinitely after completely recovering from the injection. In such cases the susceptibility coefficient $\frac{1}{\text{(days survived)}}$ was assumed to equal zero.

2. Fatigue, artificially induced in white and hooded rats by forcing them to run in motor-driven drums, apparently tends slightly to increase their resistance to subcutaneous injections of tetanus toxin. This occurs whether fatigue precedes or follows the injection of the toxin.

3. White and hooded rats, up to 185 grams in weight, are not susceptible to diphtheria toxin in doses as large as 2 c.c.

4. Fatigue, both preceding and following intraperitoneal injections of Type I pneumococcus, definitely increases the resistance of the white and hooded rats to that infection.

5. At least in the case of white and hooded rats, our results contradict the popular belief that a fatigued individual is more susceptible to disease (tetanus toxin and pneumococcus infection) than a non-fatigued individual.

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APPENDIX.

TABLE I.

SERIES A.

The M.L.D. of tetanus toxin for white and hooded rats.

Rat No.	Wt. of rat in gms.	Dose toxin in grams $\times 10^{-6}$.	Amt. toxin per gm. wt. rat $\times 10^{-7}$.	Injected.	Paralysis began.	Recovery.	Death.
1.....	135	10,000	740	Oct. 28th	—	—	2d day
2.....	155	5,000	320	"	—	—	"
3.....	170	2,500	140	"	—	—	"
4.....	205	2,500	120	"	—	—	"
5.....	205	1,000	48	"	—	—	"
6.....	140	500	35	Nov. 2d	2d day	—	"
7.....	210	500	23	Oct. 28th	"	—	3d day
8.....	139	250	18	Nov. 2d	"	—	2d "
9.....	166	125	7.5	"	"	—	3d "
10.....	153	31	2.0	"	"	—	"
11.....	154	25	1.6	Nov. 5th	"	—	"
12.....	163	17.5	1.1	"	3d day	—	4th day
13.....	103	7.0	.679	Dec. 1st	"	—	8th "
14.....	205	10.0	.486	"	"	—	4th "
15.....	120	6.0	.486	"	2d day	—	"
16.....	110	5.0	.486	"	"	—	6th "
17.....	145	7.0	.486	Dec. 7th	3d day	—	"
18.....	107	5.0	.486	"	2d "	—	4th day
19.....	181	8.75	.483	Nov. 5th	3d "	—	20th "
20.....	193	7.7	.400	Dec. 7th	"	—	5th "
21.....	145	5.8	.400	"	"	—	"
22.....	115	4.6	.400	"	4th day	—	7th day
23.....	139	5.0	.360	Dec. 14th	6th "	24th day	—
24.....	124	4.5	.360	Jan. 5th	5th "	—	11th day
25.....	123	3.7	.300	Dec. 14th	6th "	—	7th "
26.....	115	3.2	.280	Jan. 17th	3d "	—	6th "
27.....	129	3.3	.260	Jan. 31st	4th "	—	5th "
28.....	116	2.7	.250	Dec. 14th	9th "	17th day	—
29.....	125	3.1	.250	Jan. 5th	—	—	—
30.....	166	4.15	.250	Jan. 24th	8th day	32d day	—
31.....	167	4.20	.250	Feb. 9th	2d "	—	4th day
32.....	96	2.40	.250	Feb. 25th	3d "	—	20th "
33.....	124	2.90	.240	Feb. 16th	7th "	10th day	—
34.....	108	2.30	.214	Dec. 14th	4th "	—	20th day
35.....	215	4.60	.214	Jan. 5th	—	—	—
36.....	98	1.80	.180	"	5th day	12th day	—
37.....	119	1.80	.150	"	—	—	—

TABLE II.

Series B.

The relation between fatigue and susceptibility to tetanus toxin in white and hooded rats.

Exper. No.	Wt. of rat in gms.	Dose toxin in grams × 10^{-6}.	Amt. toxin per gm. wt. rat × 10^{-8}.	Date of injection.	Fatigued.			Paralysis began.	Recovery complete.	Death.	Suscepti-bility.
					Number of times.	Before inject.	After inject.				
1	120	3.0	2.5	Jan. 17th	1	—	×	2d day	32d day	—	—
	120	3.0	2.5	“	1	×	—	“	“	—	—
	112	2.8	2.5	“	0	—	—	“	“	19th day	— — — —
2	148	3.6	2.5	Jan. 24th	1	×	—	2d day	32d day	—	±
	164	4.1	2.5	“	1	—	×	“	“	—	±
	166	4.15	2.5	“	0	—	—	8th day	“	—	— — — —
3	138	3.6	2.6	Jan. 31st	1	×	—	2d day	—	6th day	—
	150	3.9	“	“	1	—	×	“	—	5th “	±
	145	3.8	“	“	1	—	×	4th day	—	7th “	—
	129	3.3	“	“	0	—	—	“	—	5th “	— — — —
4	127	3.2	2.5	Feb. 9th	3	—	×	3d day	—	5th day	—
	136	3.4	“	“	3	—	×	“	—	6th “	—
	125	3.1	“	“	3	×	—	2d day	—	5th “	—
	117	3.0	“	“	3	×	—	“	—	5th “	—
	167	4.2	“	“	0	—	—	“	—	4th “	— — — —
5	177	4.2	2.4	Feb. 16th	3	—	×	5th day	19th day	—	+
	131	3.1	“	“	3	×	—	“	12th “	—	+
	141	3.4	“	“	3	×	—	“	19th “	—	+
	124	2.9	“	“	0	—	—	7th day	10th “	—	— — — —
6	175	4.4	2.5	Feb. 25th	3	×	—	3d day	9th day	—	—
	125	3.1	“	“	3	—	×	“	—	9th day	+
	96	2.4	“	“	0	—	—	“	—	20th “	— — — —

TABLE III.

SERIES C.

The relation between fatigue and susceptibility of rats towards injections of Type I pneumococcus.

Exper. No.	Wt. of rats in gms.	Dose in c.c. of Type I pneumococcus.	No. of culture transplants.	Date of injection.	Fatigue.			Death.	Susceptibility.
					Number of times.	Before inject.	After inject.		
1	126	0.0002*	4	April 2d	1	—	×	22d day	+
	126	"	"	"	2	×	—	—	±
	126	"	"	"	0	—	—	—	----
2	134	0.002	4	April 2d	1	—	×	—	±
	115	"	"	"	2	×	—	24th day	+
	119	"	"	"	0	—	—	—	----
3	113	0.02 norm. saline	----	April 8th	1	×	—	—	----
	113	0.02	5	"	1	×	—	—	±
	105	"	"	"	1	—	×	—	±
	122	"	"	"	1	—	×	—	±
	120	"	"	"	0	—	—	—	----
4	111	0.02	1	April 15th	1	×	—	5th day	—
	137	"	"	"	1	×	—	9th "	—
	118	"	"	"	1	—	×	2d "	±
	109	"	"	"	1	—	×	10th "	—
	110	"	"	"	0	—	—	2d "	----
5	123	0.02	2	April 22d	3	×	—	5th day	—
	120	"	"	"	1	×	—	4th "	—
	138	"	"	"	1	—	×	—	—
	150	"	"	"	1	—	×	4th day	—
	123	"	"	"	0	—	—	3d "	----

* 18-hour broth culture.

TABLE III. *Series C (Continued).*

Exper. No.	Wt. of rats in gms.	Dose in c.c. of Type I pneumococcus.	No. of culture transplants.	Date of injection.	Fatigue.			Death.	Susceptibility.
					Number of times.	Before inject.	After inject.		
6	124	0.02	3	April 29th	2	×	–	7th day	–
	135	"	"	"	2	×	–	–	–
	156	"	"	"	1	–	×	6th day	–
	119	"	"	"	1	–	×	4th "	±
	117	"	"	"	0	–	–	4th "	– – – –
7	92	0.002	3	May 4th	3	×	–	6th day	–
	102	"	"	"	3	×	–	–	–
	123	"	"	"	3	–	×	–	–
	119	"	"	"	3	–	×	5th day	–
	119	"	"	"	0	–	–	3d "	– – – –
8	97	0.0002	3	May 11th	3	×	–	–	–
	114	"	"	"	3	×	–	–	–
	175	"	"	"	3	–	×	3d day	+
	140	"	"	"	3	–	×	7th "	–
	158	"	"	"	0	–	–	4th "	– – – –

TABLE III. *Series C (Continued).*

Exper. No.	Wt. of rats in gms.	Dose in c.c. of Type I pneumococcus.	No. of culture transplants.	Date of injection.	Fatigue.			Death.	Susceptib.lity.
					Number of times.	Before inject.	After inject.		
9	92	0.0002	2	May 25th	3	×	–	–	–
	122	"	"	"	3	×	–	–	–
	98	"	"	"	3	×	–	–	–
	·109	"	"	"	3	×	–	–	–
	100	"	"	"	3	×	–	–	–
	158	"	"	"	3	–	×	–	–
	151	"	"	"	3	–	×	–	–
	163	"	"	"	3	–	×	–	–
	134	"	"	"	3	–	×	5th day	+
	149	"	"	"	3	–	×	4th "	+
	168	"	"	"	3	–	×	6th "	+
	126	"	"	"	0	–	–	19th "	– – – –
	123	"	"	"	0	–	–	6th "	– – – –
10	114	0.0002	2	June 1st	3	×	–	–	–
	94	"	"	"	3	×	–	–	–
	112	"	"	"	3	×	–	–	–
	94	"	"	"	3	×	–	–	–
	87	"	"	"	3	×	–	7th day	±
	90	"	"	"	3	×	–	19th "	–
	105	"	"	"	3	–	×	–	–
	127	"	"	"	3	–	×	–	–
	120	"	"	"	3	–	×	–	–
	117	"	"	"	3	–	×	3d day	+
	122	"	"	"	3	–	×	5th "	+
	143	"	"	"	0	–	–	7th "	– – – –

TABLE III. *Series C (Continued).*

Exper. No.	Wt. of rats in gms.	Dose in c.c. of Type I pneumococcus.	No. of culture transplants.	Date of injection.	Fatigue.			Death.	Susceptibility.
					Number of times.	Before inject.	After inject.		
11	90	0.0002	2	June 8th	3	×	–	–	–
	112	"	"	"	3	×	–	–	–
	85	"	"	"	3	×	–	–	–
	80	"	"	"	3	×	–	–	–
	82	"	"	"	3	×	–	–	–
	82	"	"	"	3	×	–	–	–
	122	"	"	"	3	–	×	–	–
	152	"	"	"	3	–	×	–	–
	124	"	"	"	3	–	×	5th day	±
	118	"	"	"	3	–	×	"	±
	152	"	"	"	3	–	×	4th day	±
	95	"	"	"	0	–	–	6th "	– – – –
	102	"	"	"	0	–	–	3d "	– – – –
	97	"	"	"	0	–	–	3d "	– – – –
	90	"	"	"	0	–	–	4th "	– – – –
	170	"	"	"	0	–	–	4th "	– – – –
	104	"	"	"	0	–	–	—	– – – –

THE BACTERICIDAL ACTION OF RABBIT BILE ON CERTAIN STRAINS OF STREPTOCOCCI.*

By RUTH L. STONE.

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In 1902 Marmorek (1) discovered that certain strains of streptococci had the power to plasmolyze red blood cells, whereas other strains, apparently identical in appearance, were unable to do so. It was on this basis of hemolytic and non-hemolytic properties that Schotmüller (2), Besredka (3) and others made their first classifications of the streptococci. At this time there was a consensus of opinion that a direct relationship existed between hemolytic power and virulence; that is, the more potent the hemolytic power of the organism, the more toxic the organism. When one considers the *Streptococcus viridans*, a non-hemolytic but distinctly pathogenic organism, this original theory must be modified somewhat, but nevertheless, in general, the hemolytic streptococci are far more dangerous than the non-hemolytic types.

After a careful study of the fermentative reactions of the streptococci, it was found that they could be further divided by their specialized action upon the various sugars. This has been best shown by Holman (4), who has been able on the basis of sugar fermentations to divide each of the two original groups, hemolytic and non-hemolytic, into eight, making a grand total of sixteen types, each giving consistently different sugar reactions. Further work on these strains has shown [Dochez, Avery, and Lancefield (5)], by agglutination and other biological reactions, that not only are these groups separate entities, but that the groups themselves—for instance, the pyogenes group—may be divided into various subgroups, each giving rise, on animal immunization, to its specific antiserum. Of course, there are certain general interreactions between the strains, just as is the case with Paratyphoid A and B, but this applies only to the lower dilutions of the immune serum, the higher dilutions responding only to their specific antigen.

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* From the Department of Bacteriology and Experimental Pathology, University of California, Berkeley, California.

There is another and still more curious finding concerning the grouping of the streptococci, which we wish to present in this report. This is, briefly, that rabbit bile has a strongly bactericidal effect upon certain strains of streptococci, notably on the *Streptococcus pyogenes* (Holman), while it is absolutely innocuous to other groups of streptococci, or to other human pathogens, excepting, of course, the pneumococcus. The lytic action of bile upon the pneumococcus was first noted by Neufeld (6) in 1901, and has since been used as a routine method of identification. Beef, rabbit, or in fact almost any type of bile may be used, with the resultant complete dissolution of the pneumococci. No other type of organism is affected in this manner by bile, and even the herein-described bactericidal action toward streptococci is entirely dissimilar, for the following reasons: First, the streptococci affected are not dissolved; centrifugalization shows the bacterial bodies intact. Second, only rabbit bile is effective toward these streptococci, whereas the lysis of pneumococci is not dependent upon any particular type of bile. This bactericidal action of rabbit bile toward streptococci was first noted during a study in experimental *Streptococcus empyema* [Gay and Stone (7)]. In these experiments a strain of *Streptococcus "pyogenes"* (Holman), a hemolytic, nonmannite-fermenting organism, was used, in varying amounts. It was found that the bile from infected rabbits was always sterile, even after the administration of large amounts of culture intravenously, which killed the animal. Experiments were then undertaken to determine, if possible, the reason for this sterility.

In vitro tests, using normal sterile rabbit bile in varying dilutions and a small amount of a 24-hour serum broth culture, were made and incubated for 24 hours. Streaks were then made on blood agar plates, with a resulting sterility in the more marked concentrations of bile. The following table represents the results:

TABLE I.

10 per cent. rabbit bile—amount (in broth).	Culture Strept. "H" (24 hr.).	Growth (after 24 hr. incubation).
0.5 c.c.	0.1 c.c.	0
0.3 c.c.	0.1 c.c.	0
0.05 c.c.	0.1 c.c.	+
0.05 c.c.	0.1 c.c.	+
Broth 0.5 c.c.	0.1 c.c.	+

Thus it may be seen that 0.01 c.c. bile was sufficient to kill 0.1 c.c. of a 24-hour culture. Bile from various rabbits, both normal and immune to streptococci, was tested with this strain with the same results, the only variation being that occasionally scant growth appeared in the 0.1 c.c. dilution but never in any greater concentration. Next, various strains of *Streptococcus pyogenes* were studied. These were all killed by contact with this weak concentration of bile. Naturally, the next step was the testing of various types of bile, and the most easily obtainable were studied. Beef, sheep, pig, dog, cat, rat, guinea pig, turtle, and human bile were used, but those strains of streptococcus which were killed by such a weak concentration of rabbit bile were unaffected by any concentration of any of these other types of bile.

TABLE II.

Streptococcus pyogenes (Holman).*

Strain.	Origin.	Hem.	Killed by Bile.	Lact.	Mann.	Sal.
Holden........	Empyema	+	+	+	0	+
23/20/3........	"	+	+	+	0	+
McLanahan....	"	+	+	+	0	+
Collins........	"	+	+	+	0	+
84/18/2........	"	+	+	+	0	+
Waller........	"	+	+	+	0	+
Powers........	"	+	+	+	0	+
Speed........	"	+	+	+	0	+
Cox..........	"	+	+	+	0	+
Cratch........	"	+	+	+	0	+
Misner........	"	+	+	+	0	+
S50	Pl. fluid	+	+	+	0	+
S52	"	+	+	+	0	+
S53	Measles	+	+	+	0	+
S54	Prostatic ab.	+	+	+	0	+
01591.........	Pus	+	+	+	0	+
S7	—	+	+	+	0	+
7081..........	Equine	+	+	+	0	+
7094..........	"	+	+	+	0	+
7019..........	Canine	+	+	+	0	+
143...........	Guinea pig	+	+	+	0	+
464...........	Erysipelas	+	+	+	0	+
514...........	Empyema	+	+	+	0	+
548...........	Throat	+	+	+	0	+
S17	Empyema	+	+	+	0	+
S19	"	+	+	+	0	+
S13	"	+	+	+	0	+

Just what the reason is for this peculiar but specific action has yet to be determined. Apparently it is not due to the content of taurocholic and glycocholic acids in the bile or the relative proportion of each one that may be present. For example, rabbit bile, as we know, contains almost exclusively glycocholic acid [Hammarsten (8)]. But this is also true of pig bile, which is entirely ineffective. Nor can it be that the P_H value is of importance, since human and beef bile average about the same P_H as rabbit bile.

Having established beyond a doubt the fact that all strains of *Streptococcus pyogenes* studied (28) are killed by contact with rabbit bile, and this type of bile alone, we naturally turned to testing streptococci of all types and from varying sources, using the technic described (Table I) as a standard. For simplicity these streptococci have been divided into four groups instead of the sixteen established by Holman. These are: (1) *Streptococcus pyogenes;* (2) Hemolytic non-*pyogenes;* (3) Non-hemolytic; and (4) *Viridans* strains. The results in group 1 are as follows:

TABLE III.

*Hemolytic Non-pyogenes Streptococcus.**

Strain.	Source.	Hem.	Killed by bile.	Lact.	Man.	Sal.	Group (Holman).
S69.....	Old lab. culture	+	0	+	+	+	*infrequens*
3889.....	Cervix	+	0	+	+	+	"
198......	Gastric ulcer	+	0	+	+	+	"
326......	Sputum	+	0	+	+	+	"
3740.....	"	+	0	+	+	+	"
3582.....	Urine	+	0	+	+	+	"
188......	?	+	0	+	+	+	"
3539	Throat	+	0	+	+	+	"
3496.....	Urethra	+	0	+	+	+	"
5533.....	Equine	+	0	+	+	+	"
130......	?	+	0	+	+	+	"
3591.....	Equine	+	0	+	+	+	"
Ives.....	Empyema	+	0	+	+	+	"
R1......	Pneumonia	+	0	+	+	+	"
R2......	"	+	0	+	+	+	"
R4......	Cheese	+	0	+	+	+	"
R8......	Influenza	+	0	+	+	−	Hem. 1
Hinkle ..	Empyema	+	+	+	+	+	*infrequens*
"B".....	Throat	+	0	+	+	+	"

Of the nineteen strains here studied only one (Hinkle) was killed by bile.

* All mannite fermenters.

From the above table it may be seen that every strain of *Streptococcus pyogenes* tested, regardless of source, was killed by the action of rabbit bile. In all experiments after the first few preliminary tests, pooled bile was used, and a check with the originally tested strain ("H") was always made, in addition to the routine broth controls. It may here be noted that it made no difference in the results obtained whether plain broth, serum broth, or 1 per cent. dextrose broth cultures were used. There was no inhibition of this bactericidal action, as has been stated to be true of pneumococcus [Rosenthal and Libman (9), Nicolle and Adil-Bey (10)].

With the hemolytic non-*pyogenes* strains, entirely different results were obtained. Using the same technic as in the above table, the following results were obtained:

TABLE IV.

Non-hemolytic Streptococcus.

Strain.	Source.	Hem.	Killed by bile.	Lact.	Man.	Sal.	Group.
8754....	Bovine	0	0	+	+	+	Fecalis
207.....	Fecal	0	0	+	+	+	"
5863....	Bovine	0	0	+	+	+	"
6340....	"	0	0	+	0	+	Mitis
McL.....	Throat	0	0	+	+	+	Fecalis
X.......	Fecal	0	0	+	+	+	"
3540....	Empyema	0	0	+	+	+	"
Richards	"	0	0	+	+	+	"
227.....	Nephritis	0	0	+	+	+	"
328.....	Asthma	0	0	+	+	+	"
325.....	Sputum	0	0	+	+	+	"
3436....	"	0	0	+	+	+	"
5994....	Bovine	0	0	+	0	+	Mitis
Holman .	?	0	0	+	+	+	Fecalis
2893....	Bovine	0	0	+	0	+	Mitis
823.....	Lobar pneumonia	0	0	+	+	+	Fecalis
6582....	Bovine	0	0	+	+	+	Non-Hem. 1
6648....	Calf Scour	0	0	+	+	+	Fecalis
7019....	Distemper	0	0	+	+	+	"
7023....	Bovine	0	0	+	+	+	"
840.....	Influenza	0	0	+	+	+	Equine
Meads...	Gonorrhoea	0	0	0	0	+	Mitis
Stoddard	Abortion	0	0	+	+	+	"
495.....	Uterus	0	0	+	+	+	

These 24 non-hemolytic strains, as can be seen, are of both animal and human origin, and have been isolated from widely differing sources. None of these strains was killed by rabbit bile.

TABLE V.

Viridans strains.

Strain.	Source.	Killed by bile.	Lact.	Man.	Sal.	Group (Holman).
Meyer...	Throat	+	+	0	0	Salivarius
S26.....	"	+	+	0	0	"
S27.....	"	+	+	0	0	"
U.C.H...	"	+	+	0	+	Mitis
RH.....	Influenza	+	+	0	0	Salivarius
R12.....	"	+	+	0	+	Mitis
R5.....	Poliomyelitis	0	0	0	0	Ignavus
R13.....	Mumps	0	0	0	0	"
R16.....	Throat	0	0	0	0	"
R17.....	Ear	0	+	0	0	Salivarius
R3......	Influenza	0	+	+	0	Non-Hem. 1
R19.....	Nephritis	0	+	0	0	Salivarius
R23.....	Influenza	0	+	0	0	"
R24.....	"	0	+	0	+	Mitis

As for the strains of *Streptococcus viridans* here studied, the results are as yet unexplainable. Six out of a total of fourteen strains were killed, while the other eight were unaffected. It is interesting to note, in passing, that none of these six strains, which were killed by bile, fermented mannite, a property in common with the *pyogenes* group. From the work so far done, this seems to be the only group in which any great irregularity in this phenomenon occurs, as the other groups, with the exception of one strain in Table III, show a very clear-cut difference from *Streptococcus pyogenes.*

In order to ascertain the action of rabbit bile on other types of bacteria, about seventy different aërobes, representing the laboratory stock cultures, and a few anaërobes, were tested in a like manner. The cultures used were both animal and human pathogens. With the exception of the eight organisms listed below, all the aërobes were unaffected by the bile. According to Nichols (11), rabbit bile is bactericidal for cholera, para-typhoid, and dysentery, but this we have not been able to confirm, either by our own or by a repitition of Nichols's technique, growth having been obtained with these cultures after 24 hours' incubation in contact with bile. The eight aërobes which were killed by rabbit bile are as follows:

- *B. psittacosis*
- *B. abortus* (Bang)
- *B. cuniculi septicus*
- *B. ruber*
- *B. melonis*
- *B. juglandis*
- *B. tumefaciens*
- *B. aplanobacter*

It is of interest to note that none of these organisms are primarly human pathogens, and only three are pathogenic for animals (*abortus. cuniculi, septicus, psittacosis*). *B. ruber* is a common chromogenic air-borne organism, and the remaining four are causative factors in certain plant diseases.

The anaërobes tested gave the following results:

	Killed by bile.
B. sporogenes	0
B. welchii	0
B. centro-sporogenes	0
B. botulinus	0
B. putrificus	0
B. bifermentans	0
B. histolyticus	0

As can be seen at a glance, these anaërobes were unaffected by their contact with bile. In this instance, after the customary 24 hours' incubation, inoculation was made into Hall constricted tubes of glucose-egg albumin broth, instead of the ordinary plating out on blood agar, and in sub-cultures from bile tubes and controls (in broth) alike, good growth was obtained.

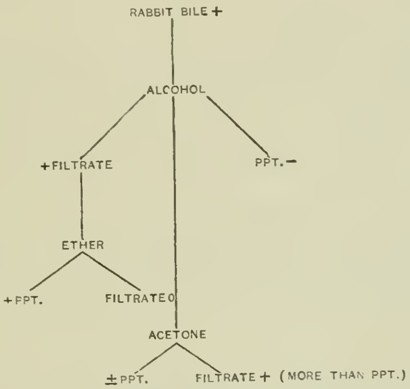
For the purpose of finding out, if possible, what substance in this specific type of bile is bactericidal for certain strains of streptococci, several further experiments were undertaken. First, it was definitely established that these organisms were really killed by the bile. and not merely attenuated. Instead of the ordinary plating test for growth in which only a large loopful of culture was used, a series of strains were tested in the usual manner, except that after the 24-hour incubation the total bile and culture (0.5 c.c.) was inoculated into a large amount (50 c.c.) of broth. No growth was obtained, showing that every organism was killed. On centrifugalization, however, the bacterial bodies were found intact.

The next question was whether the bacteria absorb this toxic substance from the bile. This was determined as follows: Having titrated and found the smallest amount of a certain lot of bile which would kill 0.1 c.c. of culture, 10 c.c. of this dilution of bile was made up and the corresponding necessary amount of culture added. This mixture of bile and culture was incubated for the usual 24 hours and then centrifugalized. The sediment was plated, to test for growth which was negative, and the supernatant fluid was then added to the sediment of the same amount of fresh culture as had been used the day previous. This centrifugalizing, for the second lot of culture added, was done in order that no further unnecessary diluant should be added. The bile was then incubated a second time, and centrifugalized as before. Plating of the sediment again gave no growth. After a third addition of culture, the power of the bile was tested by the original method of dilutions, and was found to be undiminished. Thus, it would seem that the death of the organisms is not due to absorption or adsorption of any bactericidal substance from the bile. Mere contact is apparently all that is necessary.

It was next of interest to determine what fraction of the bile was responsible for this phenomenon. Since repeated sterilization in the autoclave had no deteriorating effect on this bactericidal power, the protein fraction was precipitated out and discarded. The technique used for this fractioning of the bile was as follows: Dry sterile sand was added to a definite amount of bile, the two well mixed, and then dried and ground. This was done in order to get a greater surface for absorption. Four volumes of 95 per cent. alcohol (to one of bile) were added, a portion at a time, and the alcohol, containing whatever substances were soluble in it (bile salts, fats, pigment, etc.), was filtered off. This filtrate was measured, that an estimate might be made as to how many cubic centimeters of recovered alcohol would represent the extract from 1 c.c. of bile.

Part of this alcoholic extract was evaporated to dryness (at 60° C.) and this residue suspended in such an amount of broth that a 1/10 dilution of the original bile volume was obtained. This solution of the alcohol-soluble fraction of the bile was autoclaved and then tested for its bactericidal properties, which were found to be as great as in the original bile. The remainder of this alcoholic extract was treated with absolute ether. This gave rise to a greenish-yellow precipitate, containing the bile salts, some of the pigments, etc. In this instance, both the precipitate and filtrate were dried and resuspended, first in a small amount of water, and then further diluted with broth and autoclaved. The precipitate, or ether-insoluble fraction, was found to contain the bactericidal substance, whereas the filtrate, containing mainly fats and pigment, had no such property. From this it seems evident that this bactericidal substance is either a bile salt, or some closely allied alcohol-soluble, ether-insoluble substance. If this substance is a bile salt, it must be one that is peculiar to rabbit bile, since a mixture of taurocholic and glycocholic acids from bile, in far higher concentration than they would normally be found, caused no inhibition of growth.

Still another test was made by adding acetone to the alcoholic extract. A scanty precipitate was obtained. In this case, both the filtrate and precipitate gave evidence of slight bactericidal power, but neither one was equal to that of the alcoholic extract. This experiment was performed several times, leaving the acetone and alcohol solution together for varying intervals and using varying amounts of acetone. The results would indicate that the acetone extraction is slow, but that if sufficient time were allowed, the filtrate, or acetone-soluble fraction, would contain all the bactericidal properties. These experiments, particularly those on the alcohol and ether fractions, have been done repeatedly, with constant results. The following diagram will facilitate the understanding of these results.



+ = Bactericidal
0 = Non-bactericidal
— = Not tested

From these observations the following conlusions can be drawn:

1. Rabbit bile has a definite bactericidal effect on certain strains of streptococci and is ineffective toward others. The pyogenes group of streptococci is particularly susceptible, all the strains tested (28) being killed by rabbit bile. Other varieties of hemolytic streptococci tested (19) were unaffected, except one. There is no bactericidal or inhibitory effect upon any of the non-hemolytic streptococci. Some strains of *S. viridans* are killed, others are not affected by rabbit bile.

2. Horse, sheep, beef, dog, cat, turtle, guinea pig, and human bile exert no inhibitory action on any of these strains of streptococci.

3. The other ordinary human pathogens, with the exception of the pneumococcus, are unaffected. The action on streptococci, as described is, however, not lytic.

4. This bactericidal substance in rabbit bile is not absorbed from the bile by the bacteria. It can be used repeatedly.

5. Repeated autoclaving does not diminish this bactericidal power.

6. This bactericidal substance is located in the alcohol-soluble, ether-insoluble fraction of rabbit bile.

Many of the cultures used were obtained from the Cutter Laboratory of Berkeley, California, and also from Dr. E. C. Rosenow. Pure cultures of anaërobes were from the collection of Dr. Ivan C. Hall, of this University. This kindness and cooperation is greatly appreciated.

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THE RESULTS OF HOOKWORM DISEASE PROPHYLAXIS IN BRAZIL.

By WILSON G. SMILLIE, M.D.,

Director of the Institute of Hygiene, Faculdade de Medicina e Cirurgia, São Paulo, Brazil.

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Rural sanitation in the tropics is a problem of absorbing interest that has occupied the attention and stimulated the enthusiastic research of a large group of men. The members of the International Health Board, working in Brazil in cooperation with various federal, state, and other agencies, have devoted much effort, time and money for the past four years to the solution of some of these problems in Brazil.

We have arrived at some definite conclusions which are of special interest to Brazil, but which we believe have a wide application, and are of far-reaching importance in the practical solution of some of the sanitary problems of other tropical and sub-tropical countries.

One of the big problems in rural sanitation which has occupied our attention is hookworm disease, and after four years of labor the following questions have most urgently presented themselves:

1. Are the *results* of the intensive hookworm campaigns of permanent or temporary value?

2. If the results of the campaigns are of temporary value only, for how long a time are the benefits of the campaigns appreciable in the betterment of the health of the people?

3. At the present time we believe that the two most important factors in hookworm disease prophylaxis are intensive treatment and latrine construction.

A. Which of the two is of greater relative importance?

B. What relation should one factor bear to the other, e.g., which should have precedence?

The correct answers to these basic problems are essential if we are to carry through hookworm disease prevention to a successful conclusion. Here in Brazil, after four years of enthusiastic devotion to a cause—working under difficulties and subjected to a steady fire of criticism, one's greatest stimulus has been the self-assurance that the work could not fail, because it was based on a true knowledge of the disease. It has been extremely disheartening to learn that the results we have obtained appear to be far from satisfactory. The degree of hookworm infection by microscopic examination in Ilha do Governador, for example, before work was begun was 71.2 per cent. Four years later, after a successful intensive campaign and installation of latrines in more than 80 per cent. of the houses, the microscope shows an infection rate of 70 per cent.

Similar results are reported from other tropical countries, where similar work has been carried on for a period of years—in Ceylon, Porto Rico, various islands in the West Indies, to mention only a few.

There are three alternatives left to us in interpreting such discouraging data that have been accumulated and presented from various sources.

A. Our knowledge as to the mode of spread of hookworm disease is at fault.

B. Our methods used in combating the disease have been inefficient.

C. The data from which we are drawing our conclusions as to our failure are incomplete and misleading.

In order to meet these issues squarely, we planned a re-survey of hookworm disease in a typical community in which:

A. The International Health Board had carried an intensive campaign to a successful conclusion, more than 3 years previously.

B. The Federal Government had followed the treatment campaign with the installation of sanitary latrines, so that at the present time more than 80 per cent. of the homes have a functioning latrine.

The Ilha do Governador (or Governor's Island) was chosen for this re-survey.

A brief summary of conditions on the Ilha demonstrates clearly why it was a satisfactory field for this study. The Island is situated in the Bay of Rio de Janeiro and has a stable population of about 9,000 people. The population is widely distributed, there being only two or three villages. Many trades and occupations are represented, such as fishermen, tradesmen, factory workers, farm laborers, store keepers, truck gardeners, soldiers, sailors, etc. Most of the people are poor, living in small mud-walled houses with dirt floors.

Four years ago, when sanitary work was begun, the Island was notorious as an unsanitary place; epidemics of dysentery had caused many deaths, malaria was extremely prevalent, latrines were almost unknown, and typical cases of severe hookworm disease were to be found on every hand.

Dr. L. W. Hackett, International Health Board Director for Brazil, began the intensive hookworm campaign treatment in August, 1917. The microscopic examination of the stools of all the inhabitants, including different stations of life, all ages, and various races, showed a hookworm infection rate of 71.2 per cent. The campaign was carried to a successful conclusion in August, 1918, more than 90 per cent. of the infected individuals receiving treatment. Coincident with the campaign the Federal Government enforced sanitary regulations, requiring all homes to construct sanitary latrines. This measure was slowly enforced so that when the International Health Board withdrew, about 25 per cent. of the houses were still without latrines.

Dr. Hackett cooperated with the Federal Government at the same time in beginning anti-malaria prophylaxis, by drainage and quinine treatment of carriers, and all surface wells were filled.

When the hookworm treatment campaign ended, the Federal Government left a dispensary post in its place, which continued the enforcement of latrine construction, continued drainage operations, and maintained a consulting clinic for all types of disease.

This dispensary had an interesting regulation. This regulation refused medical consultation for any disease, until the individual had first submitted a stool specimen for examination. In case hookworm ova were found, chenopodium treatment was given. In this way the authorities hoped to cure the few refractory cases left from the campaign, and to catch any infected newcomers who might bring infection to the island after the campaign.

A few months after the campaign had closed the dispensary physician noted that many individuals who had been treated during the campaign and pronounced as cured, were again infected with hookworms. At the end of two years, on calculating his statistics, he was discouraged to find that the percentage of infection with hookworms in dispensary patients was about 70 or practically equal to that found before the campaign was carried out, and this despite the fact that more than 70 per cent. of the homes had installed sanitary latrines.

Malaria had rapidly diminished in intensity and frequency, following drainage and treatment of plasmodia carriers; dysentery and typhoid had been checked by the closing of all shallow wells, and installing a pure water supply, yet the incidence of hookworm infection had not diminished, despite a thorough, intensive treatment campaign and extensive installation of latrines.

What was the explanation of this baffling problem?

One suggestion offered was that the soil had been teeming with hookworm larvae, that the people were too poor to buy shoes, and thus, despite intensive treatment and latrines, had become re-infected, and that thus conditions had returned to those existing before the campaign was begun. If this were true, it is obvious that the destruction of larvae in the soil and wearing of shoes are not of minor importance, but are two important factors in the control of hookworm disease, and that hookworm prophylaxis is much more difficult than we had imagined.

In any case the problem could only be answered by a careful resurvey, using the worm count method as an index of the degree of infection of the people. The worm count method used was that of Darling (1). The technique has been described in various former publications (2), and need not be reported in detail. In brief it consists in choosing a group of 25–30 people, representing all ages, both sexes, various occupations, etc., and giving them a massive dose of a vermifuge which removes 98–99 per cent. of the hookworms harbored. Chenopodium, in divided dosage of 3 c.c., is usually given, preceded and followed by a heavy saline purge. All stools are saved for a period of 48 hours, the worms are picked out, counted and classified. The worm count method has a disadvantage in that only small groups of people can be examined. To be effective the group must be representative, and great care must be taken in the choice of individuals, so that a true cross-section of the community may be obtained. Young children and elderly people cannot be included because of the vigorous nature of the treatment, but this is not a serious source of error since we have already shown that hookworm infection is seldom a serious factor in the lives of the old or very young.

Experiments.

Three groups were chosen for treatment:

1. A group in one of the villages consisting of families of fishermen, shopkeepers, dock laborers, street sweepers, etc.
2. A group from the zone between the village and farm, consisting

of the families of market gardeners, day laborers, washerwomen, etc.

3. A group from the center of the island, consisting almost entirely of the families of farmers and day laborers, many of whom had no latrines or did not use the one they had.

Approximately equal numbers of men and women were chosen. There were people of all ages, of different race and color, different occupations, social status, etc. The only factors in common to all were:

1. They had lived on the island continuously since 1917.
2. They had taken hookworm treatment during the campaign three years previously.
3. They had taken no vermifuge since the campaign treatment three years previously.

RESULTS.

In all, 86 cases were carried to a successful conclusion. Four of these were controls; e.g., they had lived continuously on the Island for more than two years, and had never taken hookworm treatment.

The hookworm infection rate by the microscopic examination of the stools was as follows (Table I):

TABLE I.

Results of the microscopic examination of the stools for hookworm ova. Ilha do Governador, July, 1921.

Sanitary campaign I. H. B., 1917–1918	71.2%
Federal Government Dispensary, 1920–1921	70.0%
Our Re-survey, Institute of Hygiene, July, 1921	69.0%

The results of these microscopic examinations are apparently very discouraging, for we were able to verify the findings of the Federal Dispensary, namely, that the microscopic examination shows a diminution of only 1 or 2 per cent. in the hookworm infection rate on the Island, after four years of sanitary work.

The results obtained by the worm count method revealed quite a different picture (Table II and Chart I).

TABLE II.

Results of worm count method, as an indication of the degree of hookworm infection. Ilha do Governador, July, 1921.

Total number of hookworms harbored, 86 cases	1,242	worms
Average number of hookworms per case	14	"
Total number of hookworms harbored, 4 controls	1,299	worms
Average number of hookworms harbored per case	324	"

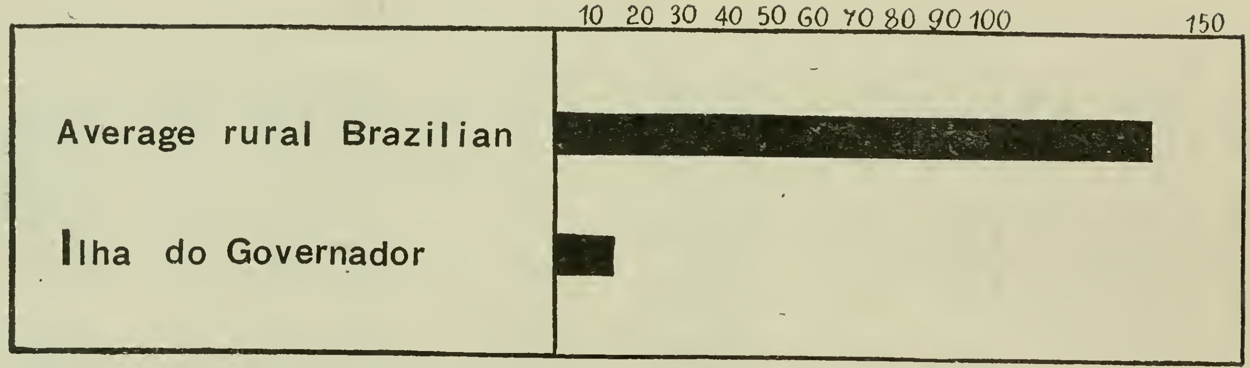

CHART I. Sanitation of Ilha do Governador—Brazil. Chart comparing the average number of hookworms harbored by residents of the Island with the number harbored by the average Brazilian.

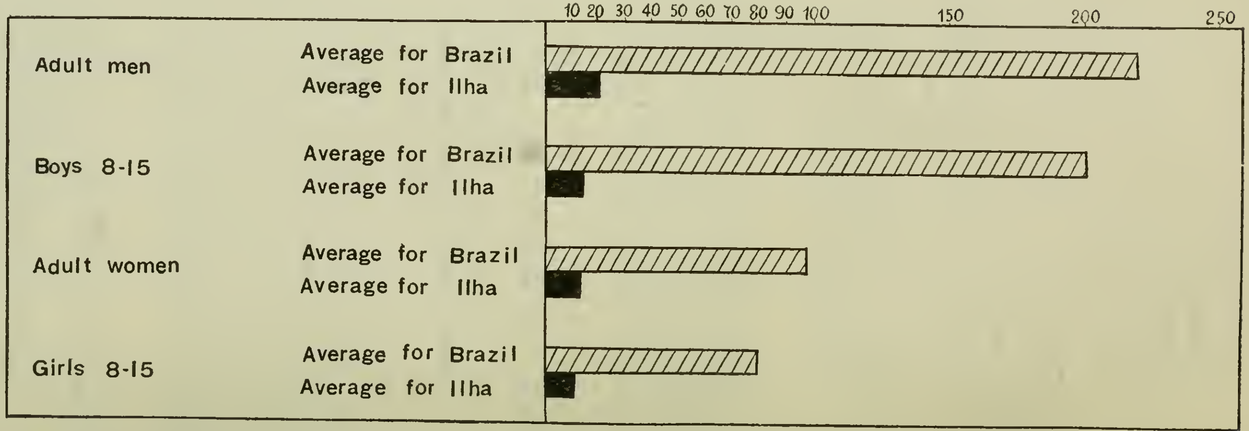

CHART II. Sanitation of Ilha do Governador—Brazil. Chart comparing average number of hookworms harbored by various groups of Ilha with number harbored by average rural Brazilian of the same age and sex.

Contained in this table is the answer to our problem. It shows that we have not been working on a false basis, our efforts have not been in vain, but on the contrary are highly satisfactory. Our mistake was in the interpretation of our data. We had fallen into the common error of forgetting the fact that hookworm infection does not mean hookworm disease. An average of 14 hookworms each, harbored by the average resident of the Island, is an insignificant degree of infection, whereas the average of 329 hookworms each, in the four controls that had never received treatment, is definite hookworm disease.

We do not know the average worm count of the Island before the treatment campaign began. Our four controls give a fair index, though all of these were adult farm laborers, and thus they followed an occupation which we have previously shown to be subject to heavy infection (3). We can fairly estimate, for purposes of comparison, that the degree of infection of the Island was probably similar to the average degree of hookworm infection for all rural Brazil. Judging by the information received from the staff who assisted in the treatment campaign, the degree of severity of hookworm disease was greater than the average for Brazil. The contrast between the present degree of infection on the Island and the degree of infection of the average rural Brazilian is strikingly shown in Table III and Chart II.

TABLE III.

Table illustrating the degree of hookworm infection of Ilha do Governador, after 4 years of sanitation, as compared with the average infection for rural Brazil.

	Number of cases.	Average age.	For Ilha do Governador.	For all rural Brazil. Same age groups.	Controls on Ilha.
Adult men........	20	29 yrs.	21.3	228	362
Boys 8–15........	15	9½ yrs.	15.0	191	216
Adult women......	28	27 yrs.	13.0	89	—
Girls 8–15........	19	10 yrs.	11.0	80	—

The maximum number of hookworms harbored by various individuals is of interest, for it is an index of the severity of infection in all the community. We have previously shown that an infection of even 50 hookworms in adults does not lower the hemoglobin index even 1 degree, whereas in children 25 or more hookworms may begin to affect the organism in a detectable way. The re-survey of the Island showed no instance of heavy infection in those cases that had been treated 3 years previously. A very few had more than an insignificant infection, as is shown by Table IV.

TABLE IV.

Table showing the maximum number of hookworms harbored, in various age and sex groups, on the Ilha do Governador.

Adult males harboring more than 50 hookworms	3 of 20
Adult females harboring more than 50 hookworms	2 of 28
Boys, 8–15 years, harboring more than 25 hookworms	4 of 15
Girls, 8–15 years, harboring more than 25 hookworms	1 of 19

Furthermore, I made a careful search of the whole Island, and in no instance could I find a single case which was obviously suffering from hookworm disease, presenting all the characteristic clinical symptoms, except in individuals that had recently come to the Island from other places and had never received hookworm treatment. I believe, therefore, that we are justified in saying that the results achieved on Ilha do Governador, after four years of hookworm disease prophylaxis, are most satisfactory. Except in the case of a few immigrants, we can truthfully say that hookworm disease as a *disease* has been wiped out on the Island. Hookworm infection still exists in about 70 per cent. of the people, but the degree of infection is of little consequence, is now diminishing, and I predict will continue to diminish.

One interesting bit of evidence that the degree of hookworm infection is diminishing on the Island is illustrated by the following facts. I noted long ago that when an individual who is infected with hookworm disease goes to a place where continued re-infection is no longer possible, as in prisons, industrial centers, institutions, etc., his degree of infection gradually decreases because the hookworms in his intestines die a natural death after a certain period of time. The females survive the males, so that on treatment of this individual, after 2 or 3 years, one will find that the patient harbors a greater proportion of female than male hookworms. Individuals who are constantly being infected, however,—cases which have never had the benefit of sanitation or treatment—harbor a slightly larger number of males than females, though the difference is very slight. This is what might be called the normal ratio of males and females.

What is true of individuals is, I believe, true of whole communities.

When proper sanitary measures are being applied so that they affect the whole community, the results should be shown after an interval of 2 or 3 years, not only by a decrease in the total number of hookworms, but also by a change in the ratio between the female and male hookworms harbored.

A comparison table of the ratio between females and males, in the cases treated on the Ilha do Governador, is suggestive (Table V).

TABLE V.

Table showing the ratio between the male and female hookworms harbored by 86 individuals on the Ilha do Governador.

	No. of cases.	Hookworms		
		Females.	Males.	Ratio between females and males.
Cases receiving benefits of sanitary measures	82	662	483	1 : 0.7
Controls—cases which have not received either treatment nor used latrines	4	669	673	1 : 1.1
Average for Brazil, 279 cases chosen at random	279	25,564	26,188	1 : 1.02

This table indicates, I believe, that re-infection with hookworms is not occurring to any extent on the Island, and that the few hookworms still harbored are decreasing from natural causes.

If then we believe that the results achieved on the Island are satisfactory we must next consider what factor was most important in bringing about this striking change in four years. To approach the question from various points of view, a division of the cases was made into four groups.

Group I.—Individuals that had received treatment during the campaign three years ago, that had also installed latrines at once and had used them—complete cases.

Group II.—Individuals that had received treatment three years ago and had not installed latrines at once, or if they did install them, used them only part of the time—complete treatment and incomplete latrines.

Group III.—Individuals that had received treatment but had not installed latrines, or if they did install them by compulsion, had never used them—complete treatment factor only.

Group IV.—Individuals that had lived on the island for more than two years and had neither received treatment nor installed latrines—controls.

A summary of the average hookworm counts of these four groups is given in Table VI and Chart III.

TABLE VI.

Comparison of the average number of hookworms harbored by various groups. Ilha do Governador, Brazil.

	Number of cases.	Average age.	Average hookworms harbored.
Group I. Treatment and latrines	44	19	2.2
Group II. Treatment, and latrines part of the time	25	20	24
Group III. Treatment, but no latrines	13	19	31
Group IV. No treatment and no latrines	4	25	324

This table demonstrates, first, that our methods of attacking hookworm disease have been sound and have produced good results; second, that the two big factors in the prophylaxis of the disease are latrine construction and intensive treatment; third, the effects of the intensive treatment campaign are clearly evident in the case of those individuals who installed no latrines (Group III), even after a period of three years. It is probable that, in part at least, these people of Group III derived protection from their neighbors who have installed latrines. On the other hand, the results show that a treatment campaign in itself should be of more than passing value. This is due to the fact that hookworm infection, as a rule, is slowly re-acquired, and rapid massive infections are rare. Fourth, the results are suggestive as to the length of time the hookworm larvae may remain in the soil in an infective stage. The soil of the Island was undoubtedly teeming with hookworm larvae when the campaign began, since latrines were almost unknown on the whole island. These larvae certainly have not survived a three- or even two-year period, for if they had, a heavy re-infection of the population would have occurred. despite latrine construction and treatment, since nearly everyone goes barefoot. The results suggest that it is the continual addition of fresh, living larvae to the soil that is a menace, and that this factor is cared for by the installation of latrines. It is unnecessary to in-

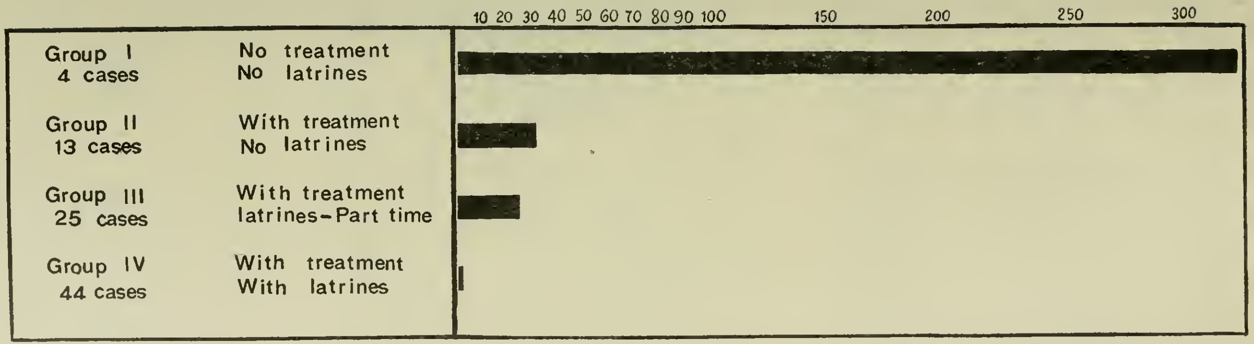

CHART III. Sanitation of Ilha do Governador—Brazil. Chart showing average number of hookworms harbored by various groups of people.

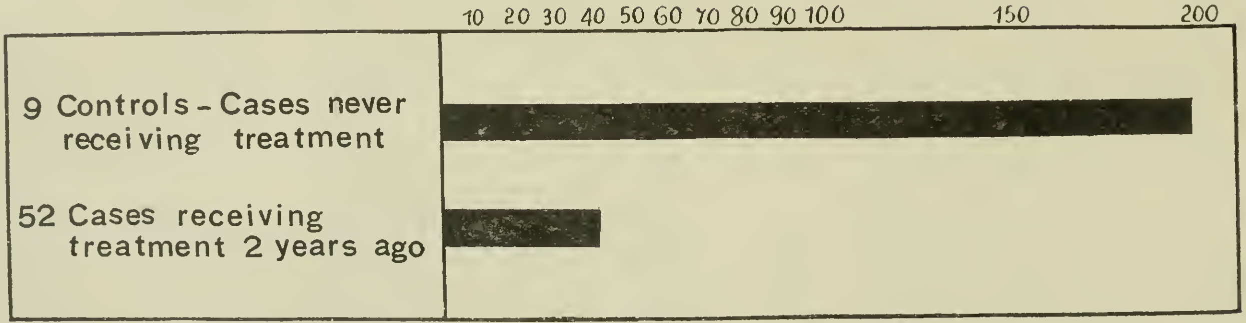

CHART IV. Sanitation of Jacarepagua—Brazil. Chart showing average number of hookworms harbored per individual in a zone of heavy infection 2 years after treatment.

stitute soil disinfection in hookworm prophylaxis, for our results show that if soil re-infection is checked, the few hookworm larvae that may survive their environment for twelve months or more are not important factors in re-infection to a degree productive of hookworm disease.

The results of the re-survey of the Ilha do Governador seem to give us clear-cut and convincing evidence that our hookworm prophylaxis program is an effective one There is one serious defect in the method of re-survey, namely, the number of cases from which we are drawing general conclusions is small.

In order that we might put our methods of procedure in hookworm prophylaxis in Brazil to a severe test, I desired to choose for re-survey another area which had all the elements necessary for the production of *heavy* hookworm infection. Such a place was found in the Municipio of Jacarépagua.

EXPERIMENTS IN A HEAVY INFECTION ZONE—JACARÉPAGUA.

The groups of people chosen for these experiments were market gardeners, most of them living on small farms, in mud houses with thatched roofs. They were very poor, using no shoes, working continuously in moist, sandy, highly cultivated soil. These conditions are typically productive of the highest degree of hookworm infection, as we have found it in Brazil. All cases, except controls, were treated two years ago by the intensive method of the International Health Board, under the direction of Dr. L. W. Hackett and the supervision of Dr. Thomas Alves. Some of them constructed latrines at once and have used them ever since, others did not.

The procedure in the re-survey of Jacarépagua was exactly as described for the Ilha do Governador.

TABLE VII.

Average number of hookworms harbored per case—Jacarépagua, Brazil. Zone of heavy infection.

	Number of cases.	Average age.	Average number of hookworms per case.
Cases receiving treatment two years ago...	52	24 years	42
Controls, cases without treatment or latrines..............................	9	20 years	198

The preliminary microscopic examination of our re-survey showed a hookworm infection index of 83 per cent. Table VII and Chart IV give a summary of the average worm counts of all the completed cases.

The results of the hookworm counts in the group of cases treated two years ago show that these individuals have re-acquired a moderate degree of re-infection, for they harbor an average of 42 hookworms each. The nine controls are heavily infected with an average of 198 hookworms each. These controls are a better index of the degree of former average infection of the zone than the controls of the island experiment, for here various ages and both sexes are represented.

The study of the distribution of the re-infection by age and sex groups of the 52 cases is illuminating. A summary is given in Table VIII and Chart V.

TABLE VIII.

Showing the degree of hookworm infection in the various age and sex groups in Jacarépagua.

	No. of cases.	Average age.	Average no. of hook-worms.	Percentage of cases harboring 50 hookworms or more (children 25 or more).
Adult men	22	33	66	40
Adult women	13	21	28	30
Boys 8–15 yrs.	10	11	36	20
Girls 8–15 yrs.	7	11	10	0

Table VIII shows that the men and boys which were treated two years ago are slowly re-acquiring infection, whereas the women and girls have a much lesser degree of re-infection. This was to be expected, for it is in accord with our previous findings that hookworm infection is acquired in a degree proportionate to the number of hours spent working in the soil. Men spend more time than boys, boys more than women, and women more than the girls.

The most interesting comparison is that of the groups, divided according to whether the individuals used latrines or not. These results are summarized in Table IX and Chart VI.

How do results differ from those of Ilha do Governador?

A. Group I, which received treatment and used latrines, shows the same results as Group I of the Island, for after a period of two years the individuals have re-acquired only an insignificant infection.

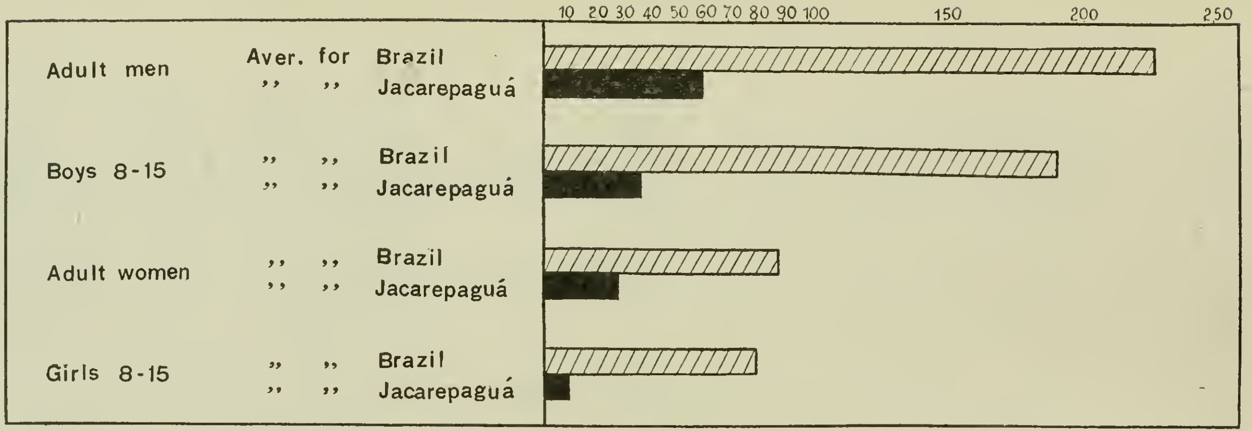

CHART V. Sanitation of Jacarepaguá—Brazil. Comparison of degree of hookworm re-infection of various age and sex groups 2 years after the campaign.

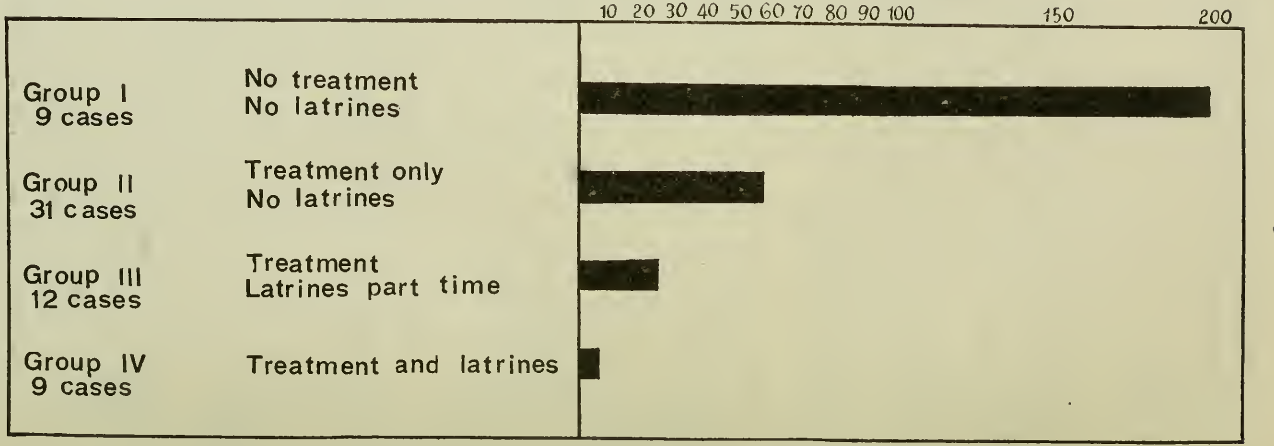

CHART VI. Sanitation of Jacarepaguá—Brazil. Comparison of average number of hookworms per case of various groups in a heavily infected zone.

B. Group II consists of individuals who received treatment two years ago, but have used latrines only part of the time and are reacquiring infection to a considerable degree.

TABLE IX.

Comparison of four groups, divided in relation to use of latrines. Jacarépagua.

	No. of cases.	Average age.	Average no. of hookworms.	Percentage of cases harboring 50 hookworms or more.
Group I. Treatment and latrines	9	37	6	0
Group II. Treatment, latrines part of the time only	12	23	25.5	16
Group III. Treatment only, no latrines	31	21	59	35
Group IV. Controls, no treatment, no latrines	9	20	198	90

C. Group III is composed of those who received treatment two years ago but have not used latrines and are acquiring infection more rapidly than Group II, though they have not yet acquired even one third as many hookworms as the controls who received no treatment two years ago.

These results prove that the intensive treatment alone, without the employment of other factors, is of demonstrable value for a period of at least two years, in a zone where every condition is favorable for a *severe* degree of hookworm infection. The results also show that latrine construction and use is all important in the permanent elimination of hookworm disease in heavily infected communities, for only in Group I can our end results be considered entirely satisfactory.

SUGGESTIONS FOR FURTHER DEVELOPMENT OF SUCCESSFUL HOOKWORM DISEASE PROPHYLAXIS.

I believe that the re-surveys of Ilha do Governador and Jacarépagua are of value not only in that they prove that our methods of attack have been productive of good results, but also because they suggest certain "follow-up" methods that may be employed with benefit in the successful prophylaxis of the disease. The results show that we should not have a set plan for all communities, but rather that our plan of campaign should be somewhat elastic, depending upon the social conditions of the people, their occupation, the density of population, per capita wealth, the degree of infection with hookworms and many other factors.

In a type community, such as the Ilha do Governador, with a population of 50 or more per square kilometer, with good means of communication with the people working at various occupations, with a per capita wealth sufficient to enable them to construct sanitary latrines, and with a hookworm infection rate of 60 to 70 per cent. by microscopic test, the following method should be economical, efficient in stamping out hookworm disease, and should be not simply an entering wedge of hygienic betterment, but a firm basis upon which to establish a future permanent rural sanitary unit.

A. The work should be begun by a general hookworm treatment campaign as at present; treating all positive cases twice and all soil workers three times, with a standard treatment of oil of chenopodium or other efficient drug.

B. Coincident with the campaign, latrine construction should be instituted. This always takes more time than the treatment campaign for obvious reasons that do not need to be detailed.

C. When the treatment campaign is completed, withdraw all but a small nucleus which shall continue fiscalization of latrine construction, and treatment of all newcomers. Certain days of the week should be devoted to general dispensary work, where anyone who comes to the clinic who has hookworm ova in the stools may be given a single chenopodium treatment. This small unit could serve as a base from which to extend permanent rural sanitary activities. Hookworm infection will not disappear, and if one uses the microscopic examination of the feces as a criterion of success, the results obtained will be most discouraging. Though *hookworm infection* will not disappear, *hookworm disease* will disappear in a very short time, and if latrine construction and maintenance is conscientiously and consistently carried forward, the end results will be all that can be desired.

In communities subjected to very heavy infection, the methods employed in hookworm prophylaxis should be somewhat different. In communitites where almost all the people are soil workers, as market gardeners or farm laborers; poor, ignorant, always barefoot, spending ten to twelve hours a day in the fields; subjected to all the factors that tend toward a heavy infection and with a hookworm index of 80 to 90 per cent. by the microscope, the plan of campaign should vary somewhat in its details.

1. The preliminary treatment campaign should be carried out as at present giving three treatments of oil of chenopodium to all soil workers.

2. The construction of latrines should accompany the treatment and should continue until 70 or 80 per cent. of the houses are sanitated. In each community this will require a considerable period of time—probably two or three years.

3. When 70 or 80 per cent. of the houses have sanitary latrines, there should then be a second hookworm treatment campaign, giving every positive case a single treatment only. Special emphasis should be placed upon treating all *soil workers.*

4. A small health unit should then be permanently maintained to fiscalize the construction and upkeep of latrines, treat newcomers, and serve as a basis for a future rural health unit.

It will be noted that I have given the treatment campaign the precedence over latrine construction in both types of community. I realize that many authorities are not in accord with this procedure, but my reasons are practical and convincing:

1. The intensive treatment campaign in itself undoubtedly alleviates a great deal of suffering and saves some lives.

2. Though of temporary value only, the re-surveys prove that its effects are clearly demonstrable even two or three years after a campaign. This two or three years' interval allows a reasonable time for latrine construction in 70 to 80 per cent. of the houses.

3. If properly carried through, the treatment campaign is a very important propaganda which demonstrates the cause and mode of transmission of hookworm disease. It is the logical and natural pre-precursor of latrine construction, for it explains why latrines are necessary and convinces the people of the importance of their daily use.

What should be the method of attacking the problem in case one is working with a community which has a high degree of hookworm infection and in which it is impossible to convince the bulk of the community of the necessity of latrine construction and use? This is not a mere hypothetical case, but an actual condition with which everyone who has worked in the tropics is familiar. In this case we have only one important weapon of attack—the treatment.

Our previous results have shown that two standard chenopodium treatments are sufficient to free the average individual from 98 per cent. of his hookworms. We may say in general that if the individual returns to the same conditions in which he acquired his infection, he will not re-acquire a heavy infection before three years. Therefore, the method applied in large plantations in the East Indies, of giving all individuals a standard chenopodium treatment once a year, may be used in the above contingency. Undoubtedly this method is an efficient prophylaxis against heavy hookworm infection in zones where latrine construction and use are not practicable. Our results show that in Brazil one standard chenopodium treatment every two years would be quite effective, even in those areas subject to heavy infection.

This method of prophylaxis yields temporary and not permanent results, it has no great educative value, and, to be effective, it requires a paternalistic form of government, such as is so commonly found on the large plantations with contract laborers.

Conclusions.

Ilha do Governador.

1. A re-survey of Ilha do Governador after four years of hookworm disease prophylaxis shows us that hookworm disease, as a disease, has disappeared from the Island. The infection index, as demonstrated by microscopic examination of the stools, has scarcely diminished at all during the period.

2. The two effective factors in this prophylaxis have been the intensive treatment campaign and the installation of latrines.

3. The individuals who took treatment, yet who had not installed latrines or who had used them only part of the time, harbor a much smaller number of hookworms than the control cases, manifesting that the benefits of the treatment alone are maintained for a period of more than three years.

Jacarépagua.

A re-survey of Jacarépagua shows that the soil workers in the heavily infected zone of Jacarépagua have a beginning re-infection with hookworms two years after an intensive treatment campaign. This re-infection is chiefly occurring in those individuals who have not installed or used latrines.

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STUDIES ON EXPERIMENTAL RICKETS. XVII.

The Effects of Diets Deficient in Calcium and in Fat-soluble A in Modifying the Histological Structure of the Bones.

By E. V. McCOLLUM, NINA SIMMONDS, and MAY KINNEY,

Department of Chemical Hygiene, School of Hygiene and Public Health, Johns Hopkins University, Baltimore,

AND

P. G. SHIPLEY and E. A. PARK,

Department of Pediatrics, Johns Hopkins University, Baltimore.

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Plates I to VII.

In a previous paper (1) we described experiments on the growing rat which were undertaken to determine the effects of diets extremely low in calcium, but which were good in other respects. The fat-soluble A* was furnished in the form of butter fat. The diets had the following composition:

Diet 2684.	
Wheat	30.0
Maize	20.0
Rice (polished)	12.0
Rolled oats	11.5
Peas	11.0
Navy beans	11.0
NaCl	1.0
$NaHCO_3$	1.5
Butter fat	2.0

Diet 2810.	
Wheat	30.0
Maize	14.5
Rice (polished)	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	9.5
Beefsteak (round)	10.0
NaCl	1.0
Dextrin	1.5
Butter fat	5.0

Ration 2684 consists of entire cereal grains and legume seeds with the exception of polished rice, supplemented with common salt, sodium bicarbonate sufficient to make a slight excess of alkaline over acid radicals, and 2 per cent. of butter fat added to supplement the diet with respect to fat-soluble A. The amount of butter fat added was not enough to bring the content of fat-soluble A up to the optimum.

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* Anti-xerophthalmic substance.

100 grams of this food mixture contained 0.0323 gram of calcium and 0.3631 gram of phosphorus.

Ration 2810 differed from ration 2684 in that it contained 10 per cent. of beefsteak, more butter fat (fat-soluble A) and no sodium bicarbonate. Its proteins were of excellent quality. Its content of fat-soluble A was approximately the optimal. It was somewhat richer in phosphorus than ration 2684. The calcium content of both diets was far below the optimal. Our experimental work with the rat indicates that a content of 0.641 gram of calcium per 100 grams of ration is very close to the optimum concentration of this element.

Ration 2810 contained in 100 grams 0.0498 gram of calcium (Ca) and 0.3900 gram of phosphorus (P). It contained only 7.7 per cent. as much as our studies indicate to be the optimal content of calcium. When 1.5 per cent. of calcium carbonate is added to this diet it becomes very satisfactory, as is shown by the fact that we have seen five successive generations of rats successfully nourished upon it.

These diets produced histological changes in the bones which have certain fundamental resemblances to, but also present certain differences from, the lesions characterizing rickets in the human being.

In the present paper we wish to describe experiments undertaken to determine the effects on the bones of a diet obtained from cereals and legume seeds unsupplemented (ration 2638); also the same diet supplemented with sodium chloride and sodium bicarbonate (Lot 2249). Our observations indicate that neither of the two salts just mentioned exert any direct influence on the structure of the bones.

DIET 2638.

Wheat	30.0
Maize	30.0
Polished rice	10.0
Rolled oats	10.0
Peas	10.0
Navy beans	10.0

DIET 2249.

Wheat	30.0
Maize	27.5
Polished rice	10.0
Rolled oats	10.0
Peas	10.0
Navy beans	10.0
NaCl	1.0
$NaHCO_3$	1.5

The diet of Lot 2638 was deficient in calcium, fat-soluble A, and a second dietary essential associated with certain fats, notably cod liver oil, provided this latter factor is a distinct entity; also, in a minor degree in sodium, chlorine and phosphorus. One hundred grams of the diet contained 0.0526 gram of calcium and 0.3646 gram of phosphorus.

The diet of Lot 2249 was deficient in the same factors as that of Lot 2638, except that it contained added sodium chloride and bi-carbonate. It contained less phosphorus than the optimum, but this deficiency was not so marked but that specially planned experiments are necessary to demonstrate that the well-being of rats can be improved by including in their diet a higher content of this element than is furnished by any ordinary seed mixture.

Diet 2638 produces histological abnormalities in the bones. These will also be considered in relation to the histological structure of the bones of animals in Lots 2810 and 2684. The latter two diets contained sufficient fat-soluble A (in butter fat) to maintain good growth and fertility, when other dietary factors are satisfactory.

Thirty-two rats were fed upon ration 2638. Some of them may be said to have received the faulty diet as soon as they were born, inasmuch as the mother animals were placed upon the ration prior to the birth of their litters, and were kept upon it throughout the period of lactation. We have shown, however, that the milk produced by a lactating rat confined to a faulty diet is a better food for her young than her diet is, as a source of nutriment for her. Up to about the age of 20 days, therefore, these young had the advantage of their lactating mothers as a factor of safety in their nutrition. Others were weaned to the faulty diet, i.e., they began to take the deficient ration as soon as they were able to eat solid food, viz.: on the 19th to 21st days. The other animals were not given the faulty ration until the 35th, 40th, or in the case of one rat, the 50th day. The rats were kept on the faulty ration for variable periods, ranging between 38 and 104 days and averaging about 71 days.

THE EFFECTS OF RATION 2638.

The animals confined to the faulty ration, 2638, grew poorly or not at all. The animals started on the faulty ration at birth (through the mother), or soon after, reached at the end of 2 months a weight, varying in a litter of four, between 25 and 37 grams. (The average weight of a rat 20 days old is 35 grams, and the average weight of a rat 2 months old is 125 grams.) The rats placed upon the ration, after weaning was complete, increased in weight for a week or more, presumably until the growth momentum derived from the previous better constituted diet had spent itself, and then remained stationary. A number of them lost weight during the last part of the experimental period, so that the final weight did not greatly exceed, or was actually less than the body weight at the beginning of the experiment. As might be inferred from the statements regarding the weight of the animals, they remained small and became greatly emaciated. The largest of them was not more than one third the size of the normal adult rat, and the smallest was scarcely larger than a mouse. None developed keratomalacia. When the animals had become so feeble, as certainly to be under the full effect of the faults in the diet, they were killed with chloroform and autopsied.

At autopsy the emaciation and stunting were striking. The incisor teeth were loose and broke, as if composed of chalk. In some animals one upper incisor had been broken off during life; in some, one or more of the incisors were irregularly curved or hypertrophied. The molar teeth were loose; in some there were cavities like those found in the teeth of human beings. The eyes appeared normal. The coat lacked the smooth glossy appearance of that of the healthy animal. The skins were adherent, a condition which seemed to be associated with the loss of subcutaneous fat. Little or no fat was found in the abdominal cavity. The thymus was atrophied. The spleen was unusually large in some animals. In the majority, however, it did not appear to be above the average size for the healthy rat of the same weight.

The skeletons of all the rats could be seen to be abnormal. Some animals showed only slight deformity, while other animals were deformed very badly. On microscopic examination a pathological condition of the skeleton was found in all animals, but this also was subject to great variation in the extent to which it was developed. We shall attempt to describe the gross and microscopic changes only sufficiently to indicate their general character, first in those animals least, then in those animals most affected.

The gross deformities of the skeleton in those rats least affected consisted in moderate degrees of enlargement of the costo-chondral junctions, without fractures of the ribs, or deformity of the thorax as a whole. There was also slight enlargement of the ends of the long bones, especially noticeable at the wrists, knees, and ankles. The femur and tibia were weak and could be cut without difficulty. The line of junction between the epiphyseal cartilage and the shaft appeared to be well defined. The cortex was thin. Microscopical examination of the adjacent ends of the femur and tibia showed that the epiphyseal cartilage was deeper in some places than others. Calcification of the cartilage was irregular. Either no intermediate zone (the rachitic transitional zone, or metaphysis) was present between the cartilage and the shaft, or else there was a narrow zone or one which was fragmentary. The trabeculae themselves were fairly numerous. The calcified portions were thin; the osteoid borders were of considerable thickness, as compared with the so-called physiological borders of osteoid, found in the bones of healthy, rapidly growing animals, but narrow as compared with the osteoid borders, found for example in rats placed on diets high in calcium and low in phosphorus, and insufficiently supplied with fat-soluble A (2). The nuclei of the bone corpuscles found in both the calcified and the osteoid portions of the trabeculae were very variable in size and shape. For the most part they were oval and slightly long, and did not appear to be especially large. Large osteoblasts with round nuclei and large cell bodies, some of them having central areas of vacuolization, were seen, however, and also osteoblasts with fusiform nuclei like those of connective tissue cells.

Trabeculae could be found which were bordered here and there or on one side by large numbers of osteoblasts, in some of which could be seen the central area of vacuolization in the cytoplasm just mentioned. The trabeculae were regularly surrounded by an investment of fibrous tissue, derived apparently from the endosteum, and in this vicinity were numbers of mononuclear cells with basophilic granulations, the presence of which in the bones of rats subjected to faulty diets low in calcium has been noted already in a previous publication (loc. cit.). The cortex was not only thin, but had a cancellous structure. The trabeculae of bone in the cortex were invested by connective tissue, and were surrounded by scattered mononuclear cells with basophilic granulations, as were the trabeculae in the spongy portion of the bone in the vicinity of the cartilage. In some rats the hyperplasia of the connective tissue about the trabeculae was so great as almost completely to fill the spaces between them, and in large part to exclude the marrow. The marrow occupying the central marrow cavity showed no very striking change. It seemed to be free from the basophilic cells found in the neighborhood of the trabeculae. Many evidences of actively progressing resorption could be found in both trabeculae and cortex.

The rats most affected by the faulty ration displayed deformities of the thorax and extremities so marked as to be visible in the living animal. The thorax was greatly flattened from side to side, and the lower part of the sternum, in consequence, was pushed forward with the production of the pigeon breast deformity. The cartilages and ribs met at sharp angles which pointed into the interior of the thorax. The costo-chondral junctions did not appear to be greatly enlarged, although greatly deformed. The posterior ends of the ribs were enlarged. The spinal column was bent. The ends of the long bones, for example, the ends of the radius and ulna at the wrist, and the femur and tibia at the knee, were enlarged. The shafts were slender, fragile. and soft. On examination of the cut surface of the femur or tibia, after longitudinal section, the epiphyseal cartilage could be seen to be of irregular depth, and to be separated from the shaft proper by a zone of white tissue. The cortex in some animals was very thin, but in others appeared thick and spongy. In these the marrow cavity was narrowed.

Before contact was established with the metaphysis all traces of columnar arrangement of the proliferative zone might or might not be lost, and its cells frequently showed no traces of any orderly arrangement. At the same time the cells themselves and their capsules expanded and became globular. The nucleus was vesicular with a fine chromatin network. The cytoplasm underwent a progressive loss of its affinity for basic dyes and the capsule became less distinct. In this condition many of the cartilage cells were incorporated in the trabeculae which formed the metaphysis. In some instances they retained their individuality, in other instances they became indistinguishable from osteoid corpuscles. Many underwent a progressive reversion to a form like that of thin fusiform connective tissue cells Many rapidly degenerated in the trabeculae. They became flattened in the direction of the long axis of the bone. The fusiform nuclei stained less deeply with haematoxylin. Nucleus and cytoplasm became less and less distinct, and finally the cell disappeared, leaving a space in the osteoid which was only to be seen by its refractive edges. Usually the cartilage cell lost the characteristics of the parent tissue almost at once after contact with the metaphyseal vessels was established, and there was only a moderate tendency either to overgrowth or to persistence of the cartilage. In other words the change of the cartilage cell into an osteoid corpuscle was very abrupt in relation to the rate of growth of the metaphysis. Long tongues of unchanged cartilage, however, persisted, extending from the epiphyseal line into the depths of the metaphysis. Unchanged cartilage cells, singly or in groups, could be identified, scattered about in the metaphyseal osteoid, especially in the periphery of the bone. Cartilage cells which retained their staining characteristics in the metaphysis were apparently those which had been protected in some way from the influence of the blood vessels with which this region is filled. The cells in the tongues of cartilage which projected below the epiphyseal line were liable to a reversion to the morphology and the staining characteristics which were shown by cells in the proliferative zone of cartilage. They were heavily stained by basic dyes. The nucleus frequently became pyknotic, and the cells were flattened, as though they were under pressure in the direction of the long axis of the bone. They had a tendency to be shaped like thin wedges or crescents, or to have a narrow elongated fusiform shape. Persistence of these tongues of cartilage was most apt to occur at the periphery of the bone. The cartilage in these animals was much more liable to disruption from invasion by blood vessels than any other that we have examined. Invasion was usually carried on by single capillary loops which forced a few cells out of their places, but in many animals large areas of cartilage were destroyed before the inroads of large tufts of small vessels which sprouted from the diaphyseal trunks. The cartilage contained no deposits of lime salts.

The metaphyses of these bones were in a large part apparently the product of the diaphysis. Besides the tongues of cartilage described above, the metaphyses contained cartilage cells isolated or in groups. These might be unchanged or in any state of transition into osteoid corpuscles or degeneration. The trabeculae of osteoid tissue which made up the greater part of the metaphysis were the result largely of the activity of osteoblasts. They were separated, one from the other, by spaces which contained the blood vessels and connective tissue. The connective tissue might be dense, or delicate, like granulation tissue. It surrounded the blood vessels and represented the replacement fibrosis of the marrow which is frequently seen in the rickets of children, and has been held to have a causal relation to disease. Fibrosis of the spaces between the trabeculae in the metaphysis was more marked in rats on these diets than in any other animals which we have examined. The fibrosis was the result of a wide fibrous investment of the trabeculae. The trabeculae in the metaphysis contained little, if any, calcified bone, and the osteoid was usually structureless. The osteoid corpuscles were as a rule larger than those found in the bones of rats on diets containing normal or slightly excessive amounts of calcium, with a low phosphate content, and they were not as widely separated from each other. Occasionally a few traces of old calcified intercellular substances were found in the metaphysis.

There was no sharp line of demarkation between the diaphysis and the metaphysis. The trabeculae of the spongiosa were surrounded by a broad zone of osteoid tissue which was identical with that of the metaphysis except that it had a lamellar structure. There were abundant evidences of resorption to be seen. The osteoblasts which surround the osteoid investment of the trabeculae were usually endothelioid in form and were in a continuous layer. In some bones many mast cells were to be found in the connective tissue between the trabeculae. In sections which had been stained with thionin the processes of the bone corpuscles in calcified bone were brilliantly colored, but nothing of the sort could be made out in connection with the same cells in the osteoid. The cortex of the shaft was represented by a lattice-like structure of lightly calcified tissue, with much osteoid, and it was pierced by large numbers of blood vessels which entered the bone from the subperiosteal-plexus—the pseudo-perforating canals described by Pommer (3). Osteoid tissue was present in the epiphyseal nuclei of ossification, in amounts equivalent to those found in the shaft. All the bones of the skeleton were affected to a greater or less extent, even the vertebrae, the small bones and the calvarium. In the ribs the changes were greatly exaggerated, and in many animals the ribs were made up entirely of osteoid tissue, in which scarcely a trace of lime salts could be found. There were evidences of many fractures in the ribs, and although these had healed with the formation of the exuberant callus there was no calcification of any of the fracture sites.

Discussion.

Diet 2638 did not produce a constant pathological condition, but one varying within rather wide limits. The lesions produced were, in a qualifying sense, always the same, and may be said to have been of a rachitic nature. They were so developed in some animals as to form a pathological picture exactly comparable to that found in rickets, while in other rats they were so poorly developed as to form a picture which may be said to have stood mid-way between rickets, on the one hand, and osteoporosis on the other. The pathological condition in the bones of the most severely affected animals fulfilled the ordinary criteria regarded as essential for the diagnosis of advanced rickets in the human being, (1) Absence or irregularity in the calcification of the provisional zone of the epiphyseal cartilage. (2) Irregularity in the depth of the epiphyseal cartilage, the result of the irregular destruction and absorption of the cartilage by the marrow elements. (3) The formation of a metaphysis or transitional zone, largely or entirely free from calcium, composed of a mixture of elements of the cartilage and the shaft, (4) The formation of osteoid borders about the calcium-containing trabeculae of the shaft.

The pathological condition in the most severe cases caused by faulty ration 2638, however, differed in certain details from that usually found in the human subject of rickets:

1. The metaphysis had a more orderly arrangement. It was composed chiefly of broad trabeculae which, though entirely uncalcified, were disposed in a more or less regular manner. In the child with advanced rickets the metaphysis is a mixture of cartilaginous and diaphyseal elements intermingled in the utmost confusion.
2. The cartilage seemed to undergo a more sudden transformation into osteoid than in the human subject. In the rachitic child the process of transformation of cartilage into osteoid. and the degeneration of the cartilage seems to go on slowly. There were found, however, scattered through the metaphysis cartilage cells in all stages of degeneration or reversion to early states of development, or metaplasia into osseous or connective tissue elements.
3. The evidences of resorptive activity were especially marked.
4. Cells filled with basophilic granules lay scattered about in the immediate vicinity of the trabeculae.

In the last two respects the bones of the rats on diet 2638 were like those found in animals after feeding diets 2684 and 2810. These last mentioned diets were characterized also by a low calcium content and an amount of phosphorus slightly above the lower limit of the optimal for growth and maintenance in the rat. The animals on diets 2684 and 2810 were, however, supplied with a much larger amount of the fat-soluble A in the form of butter fat—2–5 per cent. of the total ration. The cartilage of certain rats on diet 2638, however, was disorganized to a much greater extent than those of animals on 2684 and 2810.

The condition commonly found in the bones in human rickets was more closely imitated in the bones of rats maintained on our diets 3127, 3143, and 3133, which were low in their content of phosphorus and insufficiently supplied with the fat-soluble A, but which contained optimal or slightly excessive amounts of calcium salts (3). Comparison of the bones of animals, fed diet 3133, with those fed diet 2638, shows that in the reaction excited by feeding the former type of diet the growth and persistence of cartilage was much more exaggerated. Diets of the type of 2638 induce in the most severe cases a pathological change, in the course of which destruction, both of bone and cartilage, is much more pronounced. Certainly, however, as is demonstrated by the illustrations, the pathological condition produced by ration 2638 can be said to have borne fundamental resemblances to rickets, as that disease ordinarily manifests itself in the bones of the child, and must be considered as closely related to the rickets of the human being.

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3. Pommer.
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EXPLANATION OF PLATES.

Plates I to V.

Figs. 1, 2, 3, 4 and 5. These photomicrographs were taken of sections from the distal ends of the femura of rats which had been given the diet of lot 2638. These bones show every gradation between severe rickets (Fig. 1) and a condition in which osteoporosis is almost completely developed (Fig. 5), with intense resorption and complete calcification of the proliferative cartilage.

Plate VI.

Fig. 6. Higher magnification than the preceding figures, to show the persistence of the cartilage cells, their degeneration and the invasion of the cartilage by blood vessels. Leitz achromatic objective No. 3. No ocular.

Fig. 7. High power photomicrograph showing osteoid tissue about the trabeculae of bone. Leitz achromatic objective No. 6. No ocular.

Plate VII.

Fig. 8. This picture shows in detail the " degeneration " of the cartilage cells and their direct transformation into osteoid corpuscles. Leitz achromatic objective No. 6. No ocular.

Fig. 9. Large cells with basophilic granulations (connective tissue mast cells) clustered about trabeculae which show marked signs of resorption. Leitz achromatic objective No. 6. No ocular.

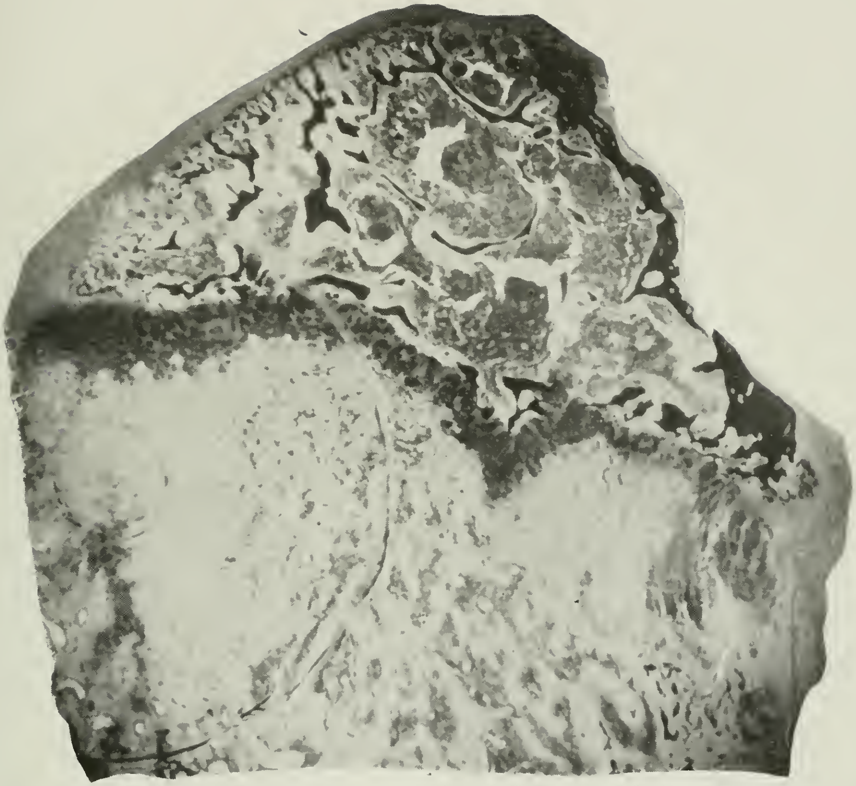

Fig. 1.

PLATE I.

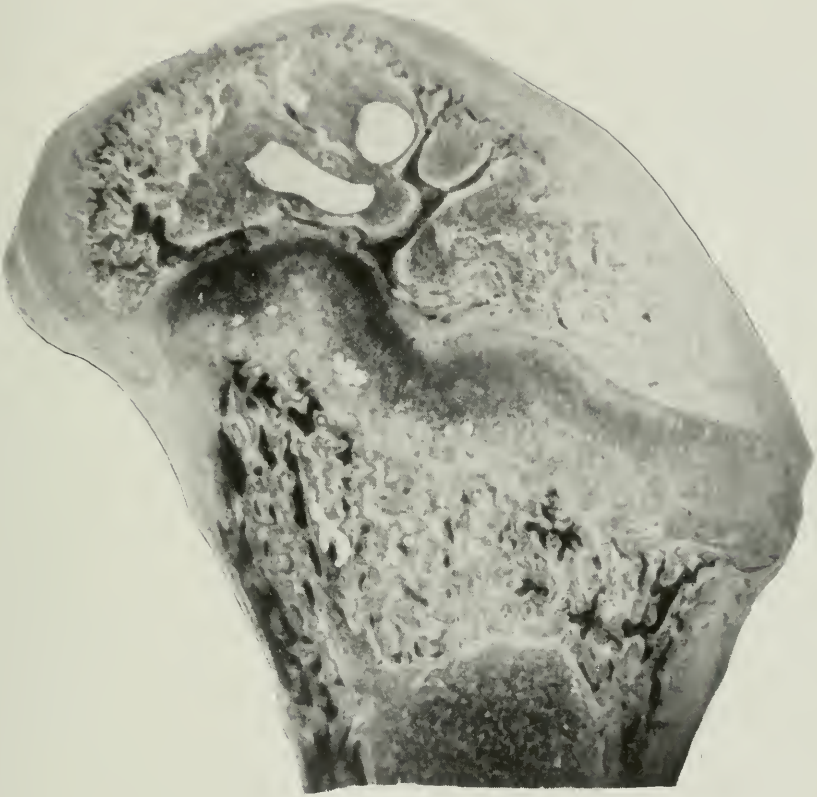

Fig. 2.

PLATE II.

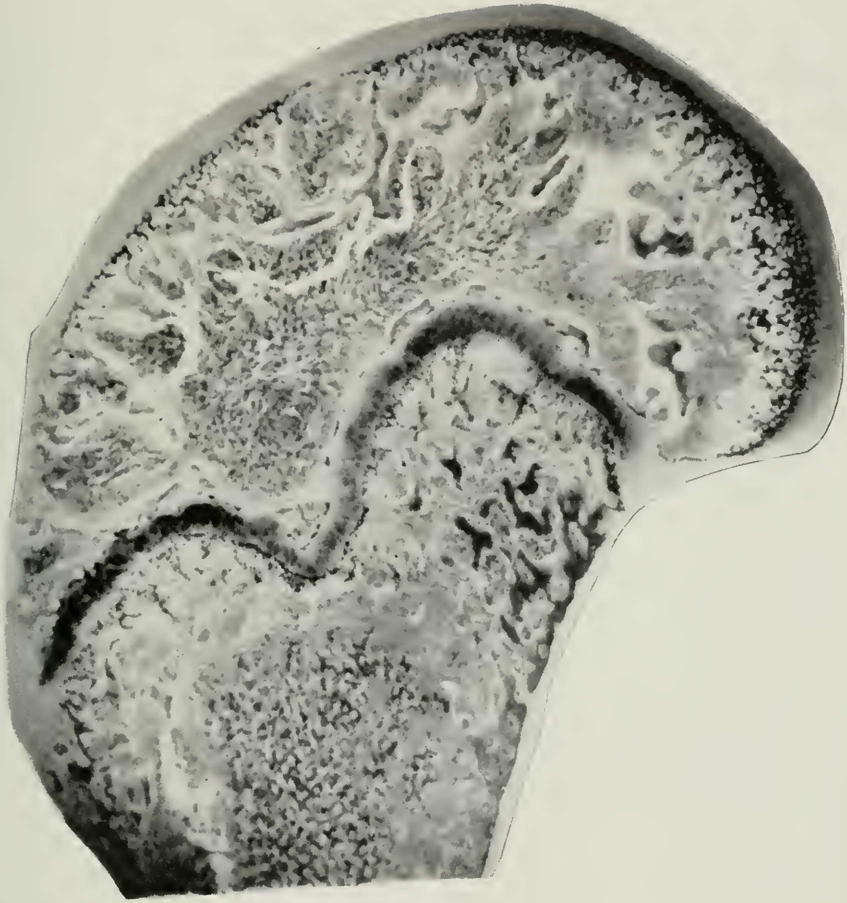

Fig. 3.

PLATE III.

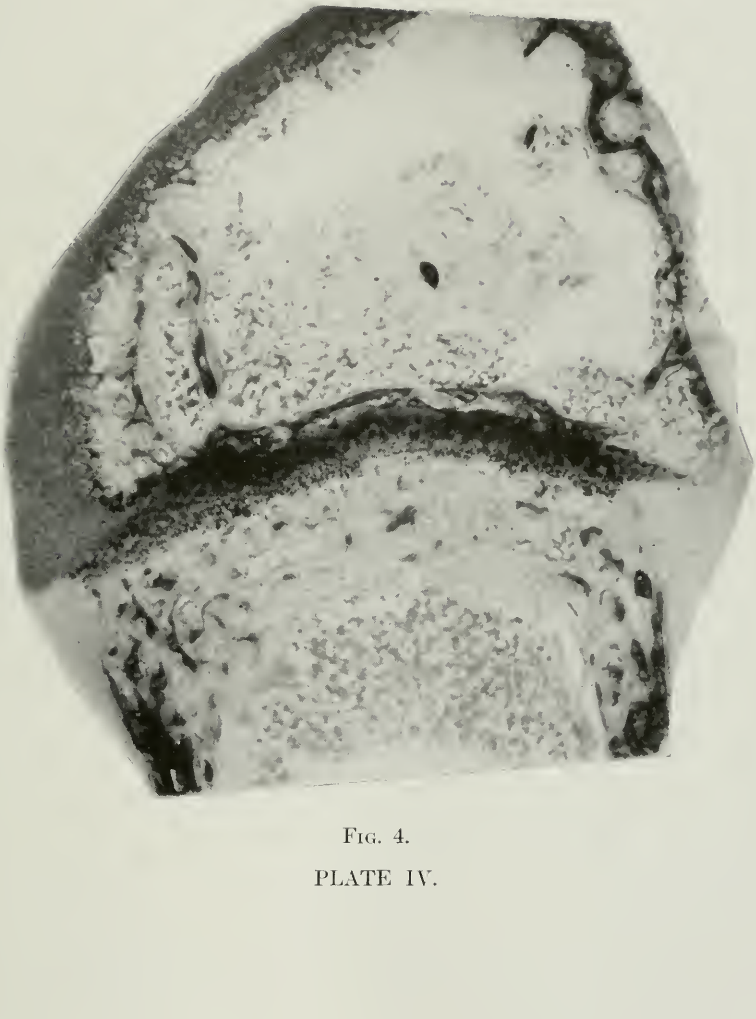

Fig. 4.

PLATE IV.

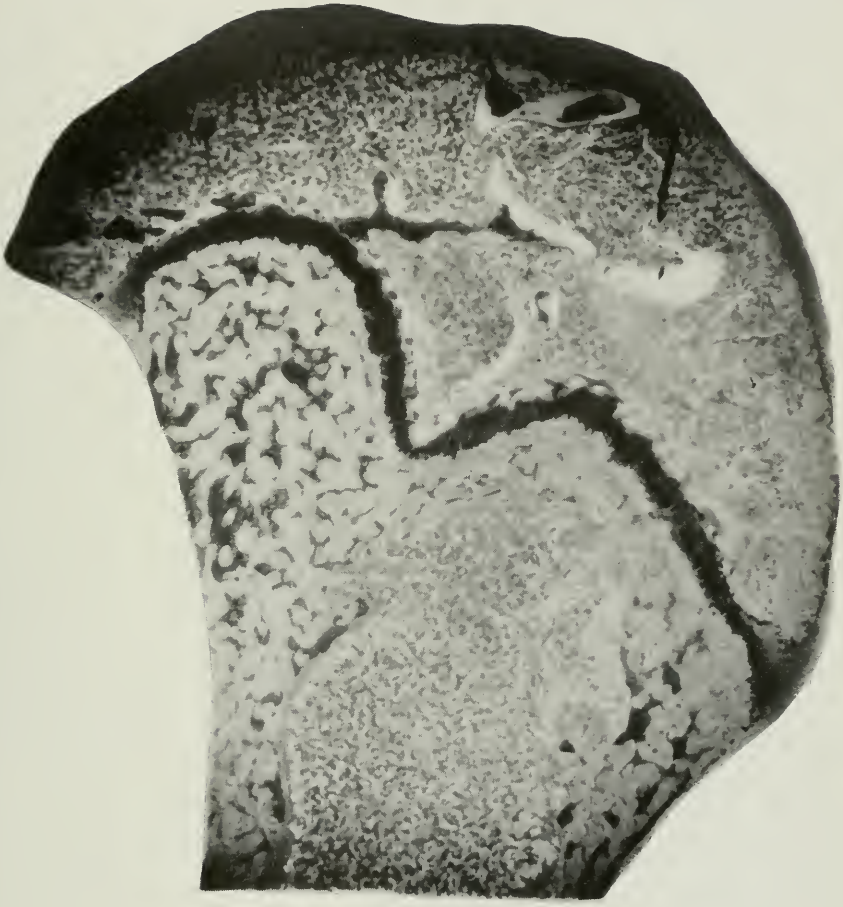

Fig. 5.

PLATE V.

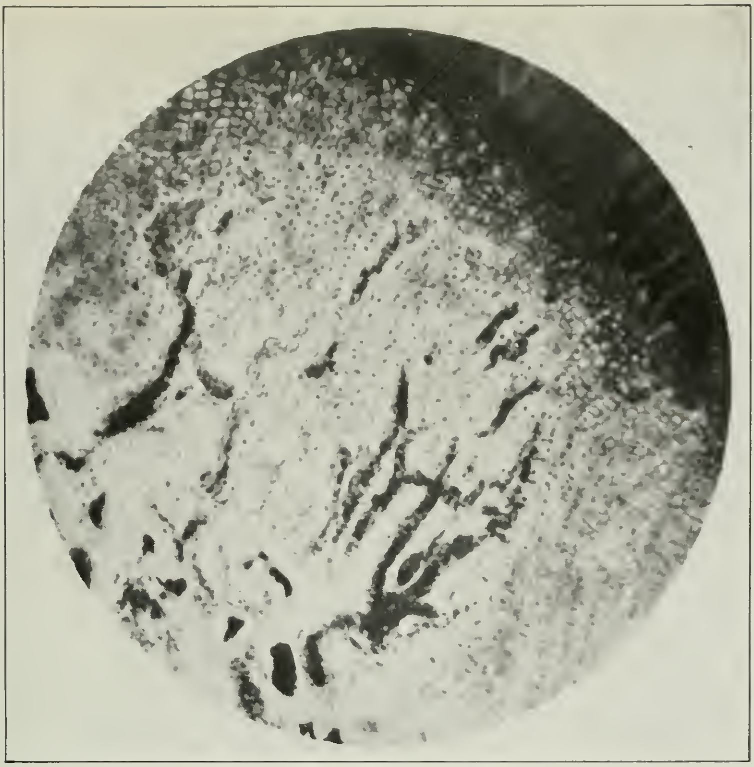

Fig. 6.

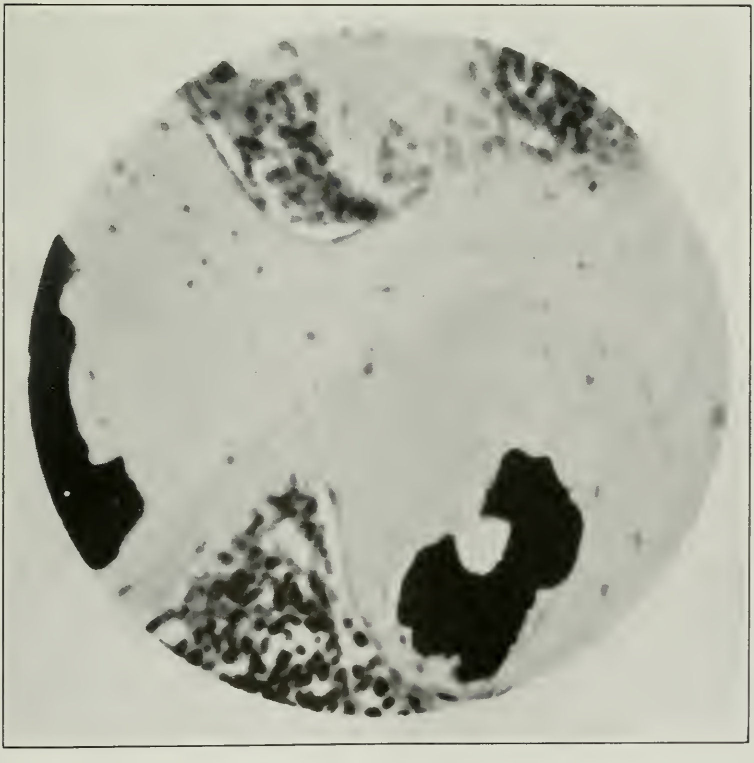

Fig. 7.

PLATE VI.

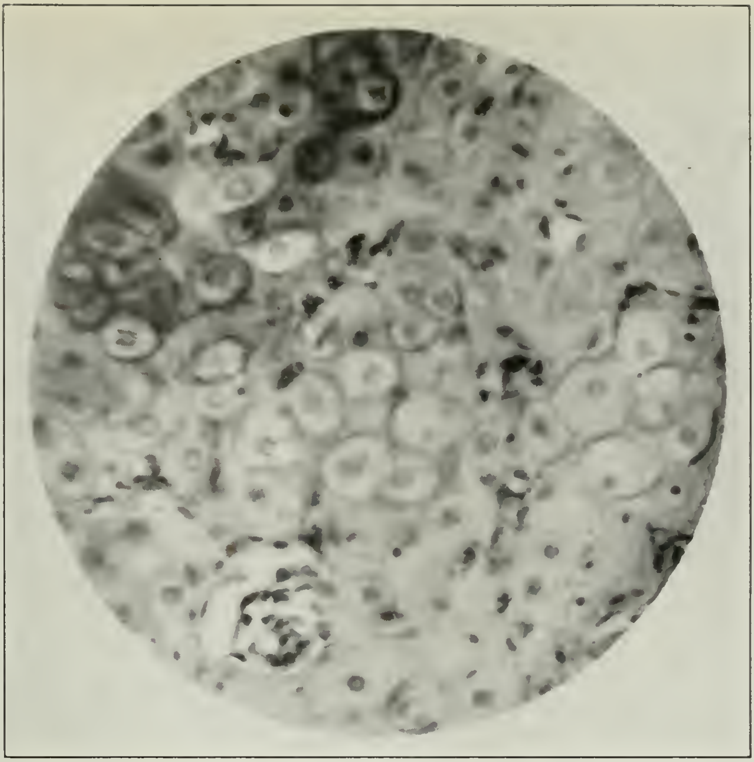

Fig. 8.

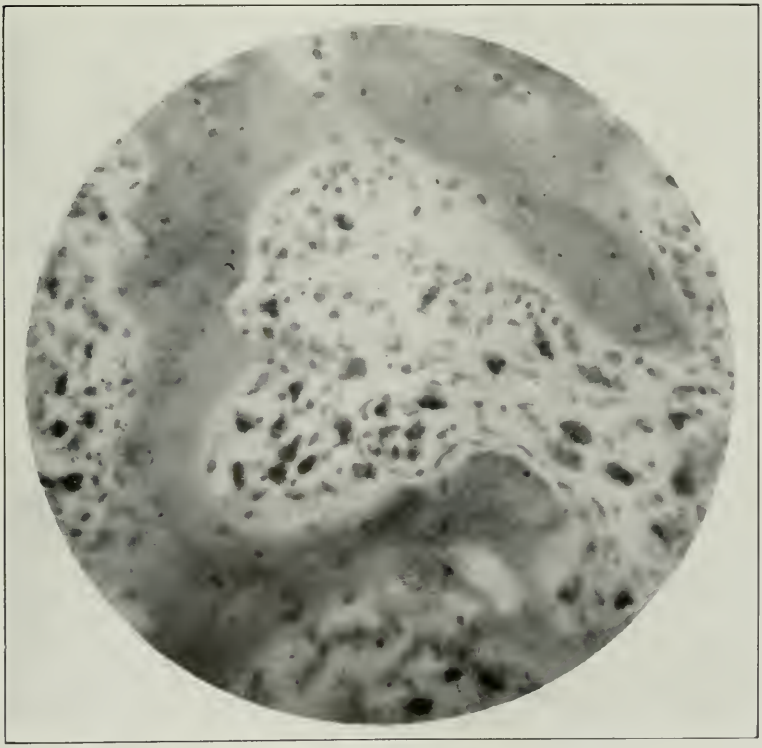

Fig. 9.

PLATE VII.

INVESTIGATIONS ON THE CONTROL OF HOOKWORM DISEASE. VI.

A Study of the Effect of Hookworm Control Measures on Soil Pollution and Infestation in a Sugar Estate.*

By WILLIAM W. CORT, Ph.D., and GEORGE C. PAYNE, M.D.

(Received for publication, December 13, 1921.)

Introduction.

In considering the aetiology of hookworm disease, the fact is soon apparent that the factors involved in the passage of the infestation from one individual to another are very complex. In order to ascertain accurately how the disease is spread in any given area it is necessary to consider (1) the extent and degree of the infestation of the people, (2) the prevalence of soil pollution, (3) the distribution of soil infestation and (4) the habits of the people with regard to possible effective contacts with the places of soil infestation. The investigation, the results of which will be given in this paper, is an epidemiologic study of a heavily infested area of a sugar estate. In this study an attempt was made to investigate all of the above-mentioned factors, both before and after the application of control measures, in order to determine the exact sources of the human infestation, and to learn the effect on human infestation, soil pollution and soil infestation of the control measures used.

Description of the area of the sugar estate chosen for study.

The area chosen for this study (see map and Fig. 1) was a part of a sugar estate about one mile east of Prince's Town, Trinidad, British West Indies, and consisted of a set of thirteen barracks and their immediate surroundings. The barracks were on a hill and formed a definite unit set off from neighboring habitations. The barracks with their yards were bounded on the south by the main road, on the east by an estate road, on the north by a steeply sloping meadow and on the west by the edge of a sugar cane field, part of which was included in the study.

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* This paper is the sixth of the series on the hookworm investigations of the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University, carried on with the cooperation of the International Health Board, Rockefeller Foundation.

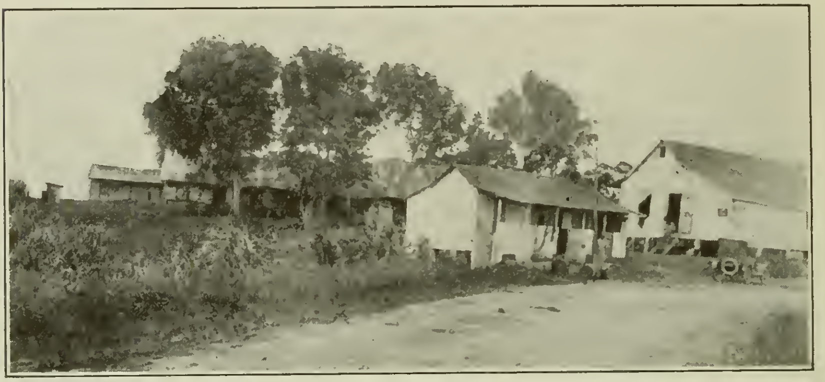


FIG. 1. View taken from the road, showing the relation of the barracks to the edge of the cane field.

The slopes in this area are quite steep. It is separated into two parts by a ridge running from north to south, just east of barracks 2, 11 and 13. The part east of this ridge drains to the east sharply, and that to the west drains from three directions into a gulley running between barracks 1 and 13 and continuing into the cane field. At the beginning of the work, the drainage around the houses was very poor, but it was later improved by the cleaning out of the old ditches and

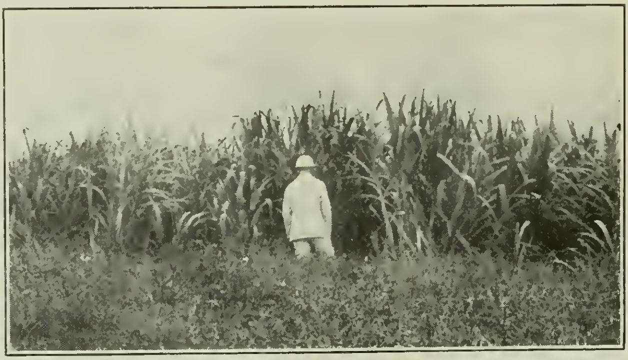
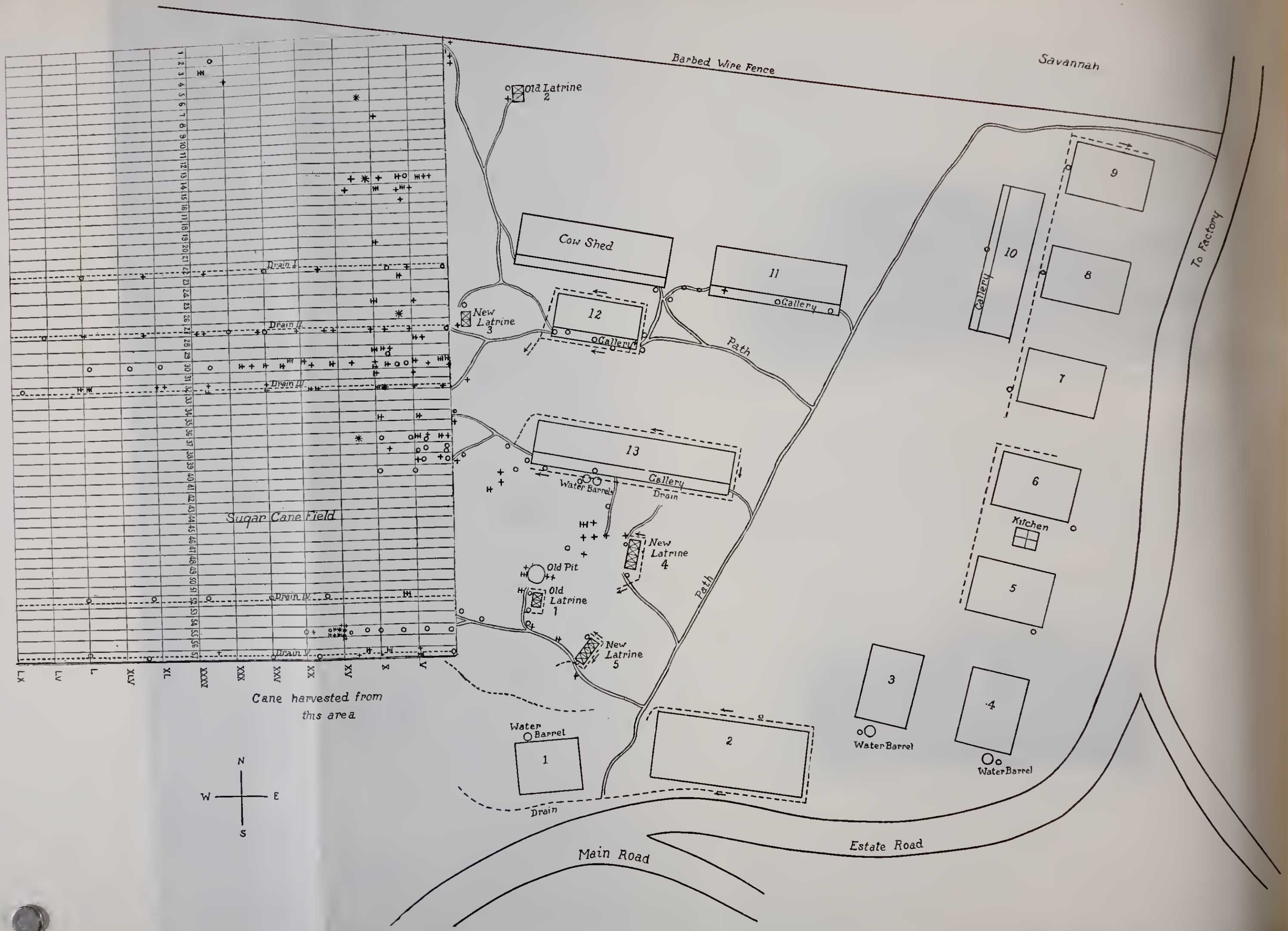


FIG. 2. View showing the size of the cane at the beginning of the work and the width of the rows.

Map of Area of Sugar Estate.



the digging of new ones. The soil was a clay loam which adhered readily to the feet. After a rain the whole area, even in the paths, was very muddy.

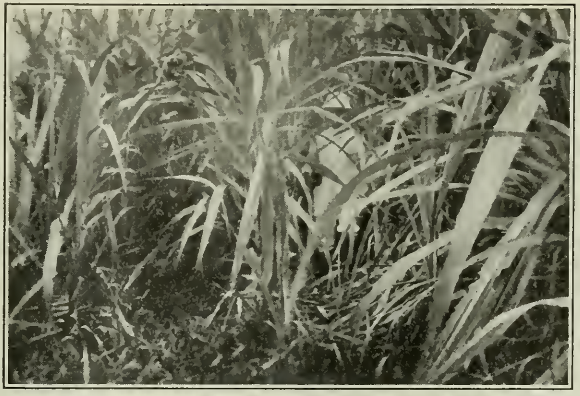


FIG. 3. View showing the kind of cover afforded by the cane.

There were a few trees around the barracks, and dense grass covered all the area. There was, however, no cover except that afforded by the cane field to the west. This field was not fenced and its wide rows were easily entered (see Figs. 2 and 3).

At the beginning of our investigation the buildings consisted of thirteen barracks, a large cow shed, a small shelter for cattle which was soon torn down, and two latrines of two units each. Later three more latrines were built, making a total of five latrines of fourteen units. The location of these buildings is shown on the map. The barracks were of two general types, as shown by Figs. 1 and 4. Those along the two roads were new and in good condition, but numbers 11,

EXPLANATION OF MAP OF AREA OF SUGAR ESTATE.

In this map the barracks are numbered from 1 to 13. In the cane field to the left on the map the parallel lines indicate the rows, which are numbered consecutively from north to south. The number of yards from the edge of the cane field is indicated by the Roman numerals along drain 5. The paths are shown by two parallel lines and the drains by dotted lines. The position of every soil sample taken from the area and the number of infective hookworm larvae present in the samples are shown by the following signs:

No larvae = 0　　11 — 30 = ±　　100 — = *
1 — 10 = +　　31 — 100 = $\overline{\underline{+}}$

12 and 13 were in the last stages of dilapidation, as shown by the photograph of barrack 13 (see Fig. 4). All had wooden floors, but the open galleries were floored with packed clay.

Animals ranging over this area were almost entirely limited to

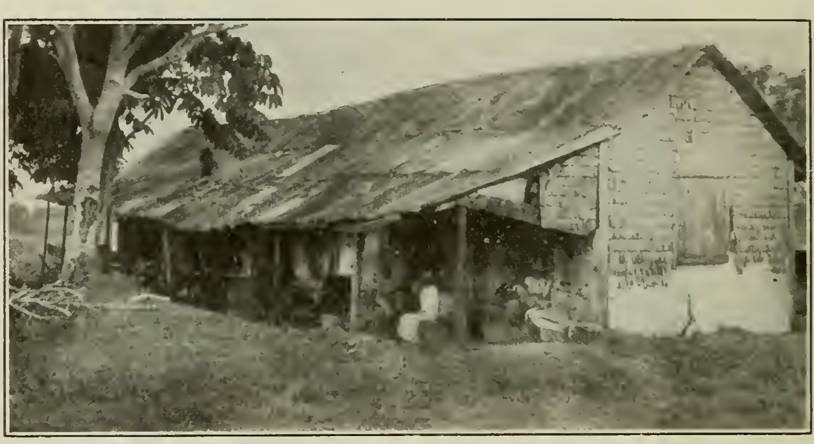


FIG. 4. Barrack 13.

FIG. 5. A mother and three children from barrack 13.

cattle and goats, which were staked out to graze on the luxurious grass, but did not have free access to the cane.

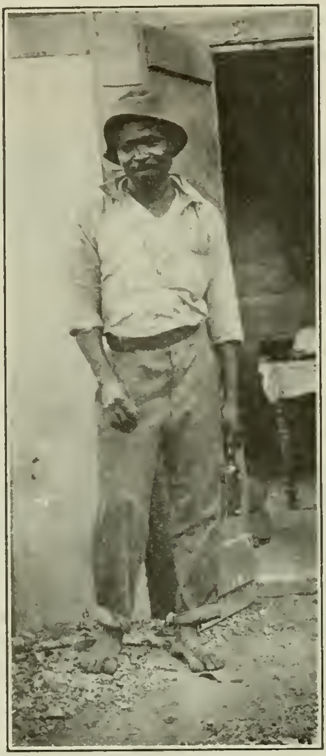


FIG. 6. Negro laborer of better type, showing bare feet covered with mud.

In all 146 people, the estate laborers and their families, lived in the thirteen barracks. Of these, 42 were negroes and 104 East Indians. There were 42 children under 12 years of age, and of the remaining 104 individuals 58 were males. Practically all the people, except the very young children, the aged and those incapacitated by illness, worked in the fields. This group of people (see Figs. 5, 6, 7 and 8) was characteristic of the type of estate laborers found in Trinidad.

INFESTATION OF THE PEOPLE.

To discover the individuals infested with hookworm disease in this area the technic of diagnosis commonly used in the hookworm campaigns of the International Health Board in the West Indies (Howard, 1919) was used. This method consists of the examination of two smears and the centrifugation of the negatives, followed by the examination of three smears of the sediment before a sample is finally declared to be negative. In order to make it possible to compare the mass infestation before and after treatment an attempt was made to roughly estimate the output of eggs. A record was kept of whether the particular specimen was positive on the first or the second smear or after centrifugation. If the specimen was positive on the first smear, a count of the number of eggs present in a smear 22 mm. by 16 mm. was made. In this way a rough comparative estimate was made of the intensity of the infestation.

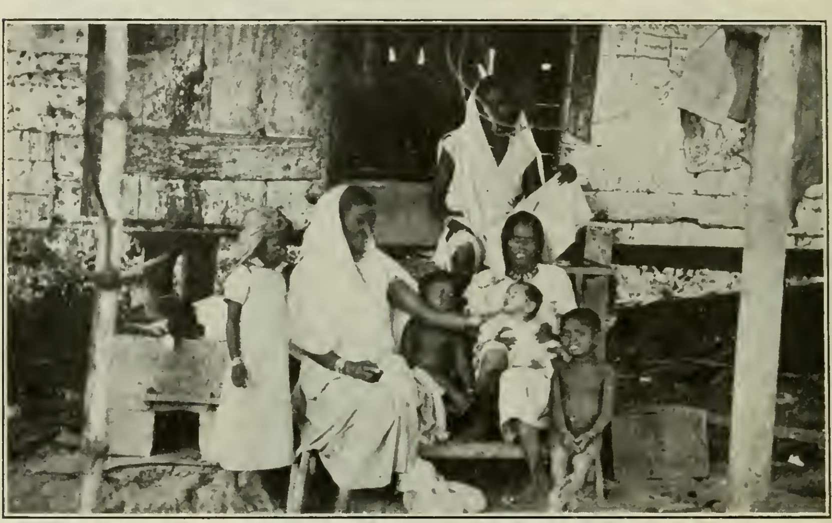


FIG. 7. Group of people from barrack 13. The little girl to the left was in a serious condition from hookworm disease complicated with malaria.

We realize of course that this method is much less accurate in estimating the reduction of infestation than the worm count method used by Darling and his associates (Darling, Barber and Hacker, 1920). Baermann (1917*a*) used the method of comparative haemoglobin estimates to test the result of his treatments in reducing infestation in the epidemiologic studies which he made in Sumatra. Since it was not possible to use either of these methods under the conditions of our investigation, we had to be content with a less accurate measure of the reduction of human infestation.

Out of the 146 people living in the area, 142 were examined for hookworm disease. Of these 117 were found to be positive and 25 were negative. Of the positives, 98 were diagnosed on the first smear with an average of 24 eggs per smear, 15 were diagnosed positive on the second smear, and 3 after centrifugation. A comparison of the negroes and East Indians showed no significant difference in the degree of infestation. Also a comparison of men and women showed nothing striking. Children under 5 years of age were found to be much less heavily infested than the rest of the population, for out of 18 children below this age only 7 were found to be positive.

FIG. 8. One of the old men from barrack 11.

The treatment of the people of this area started on July 22d. Each patient was given three treatments at intervals of a week before re-examination, which was made at least ten days after the last treatment. Each treatment consisted of a preliminary purge of magnesium sulphate administered at bed time, followed by a small dose of thymol at 6 A.M. the next day and a dose of oil of chenopodium, accompanied by a saline purge two hours later. Each dose of medicine was administered by a nurse, and the patients were kept under observation for several hours after treatment. The dosage used was on the basis of 30 grains of thymol or 30 minims of oil of chenopodium for a strong adult negro. Treatments were prescribed for each patient after a physical examination.

The record of the re-examinations, after treatment, shows a large number of negatives and a considerable reduction in the degree of infestation of those still found to be positive

Of the 117 individuals found positive in the preliminary examinations 110 were treated, 102 being given three treatments, 3 two and 5 only one. Of the 110 individuals treated 96 were re-examined after an interval of at least two weeks, to determine the effect of treatment. Of these 96 re-examinations 52 were negative. Of the positives 24 were found on the first slide with an average of 2.3 eggs per slide, 13 were found on the second slide and 7 after centrifugation. A comparison of these results with the preliminary examinations showed that a great reduction of mass infestation was brought about by the treatment.

Soil pollution.

It was the amount and general distribution of soil pollution over this area that first suggested that it would make a good place for an intensive study. When the first visit was made on June 22 there were two latrines (latrines No. 1 and 2), of the type shown in Fig. 9, for the use of the whole population. One of these latrines was full and was in an indescribably filthy condition. Feces was smeared all over the floor and in front and along the path leading from it. The other was not so bad, because it was apparently but little used. With only two latrines for a total population of 146, it is not surprising that soil pollution was found in every accessible place. For the purpose of discussion, the soil pollution conditions will be described first for the areas around the houses and yards, and second in the cane field, west of the barracks. At different times fresh stools were found in almost every situation around the barracks and in the yards. A special series of observations was made around barrack 13. Here on a mound, just two yards from the corner of the house, fresh stools were found day after day, sometimes as many as four on a day. Also in the area in front of this barrack stools could always be found.

In the vicinity of the latrines, especially around No. 1, there were always found stools. Soil pollution was common in the grass along the edge of the cane and in the grass in various places in the yards. Stools were even found in the paths and under the houses. The children were undoubtedly responsible for most of the pollution near the houses. The people said that it was impossible to control pollution by the children, which can readily be understood when we consider the conditions under which they live. The pollution in the yards was for the most part scattered and not concentrated into definite spots, which were visited day after day.

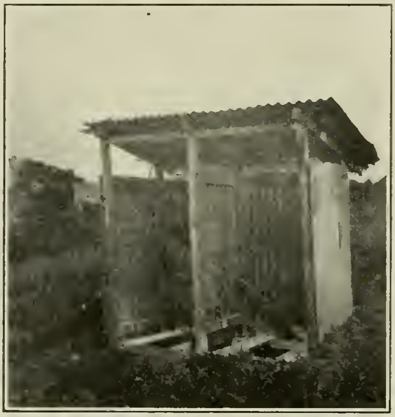


FIG. 9. Latrine number 2 at the time of the first visit to the area.

In the sugar-cane field, west of the barracks, conditions were studied more intensively, and pollution surveys were made during the whole course of our investigations in this area. The method of making a pollution survey in the cane was very simple. Charts were made indicating the position of the rows, the drains and the distances by yards from the edge near the barracks up to thirty-five yards into the cane. The persons making the survey then walked through this portion of each row and indicated on the chart the position of each stool found. The distance of the stools from the edge was measured by the number of paces. Fifteen such surveys were made of the northern half of the strip of cane bounding the area on the west (see map), of which seven were made before the construction of new latrines and the educational work had appreciably influenced the pollution.

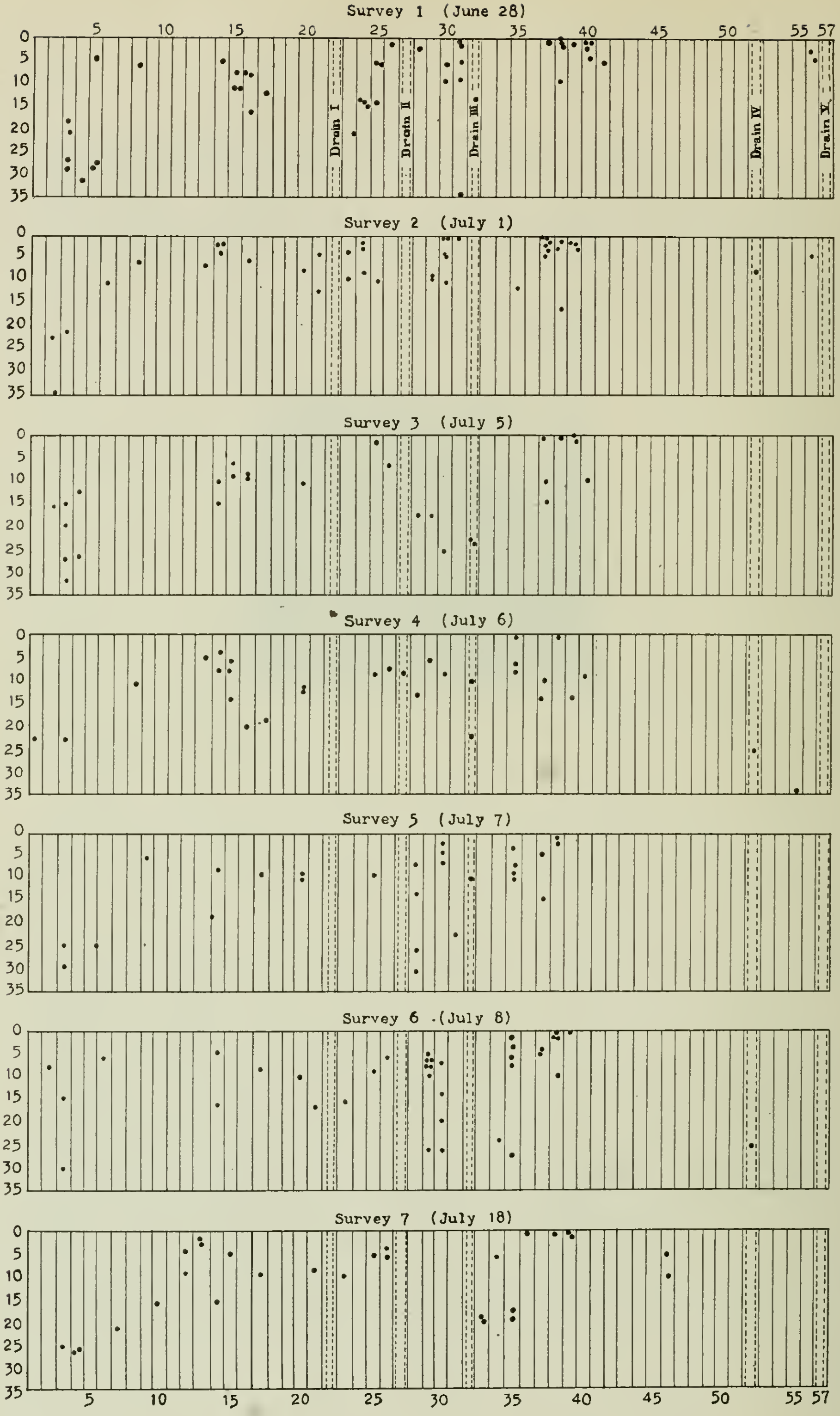
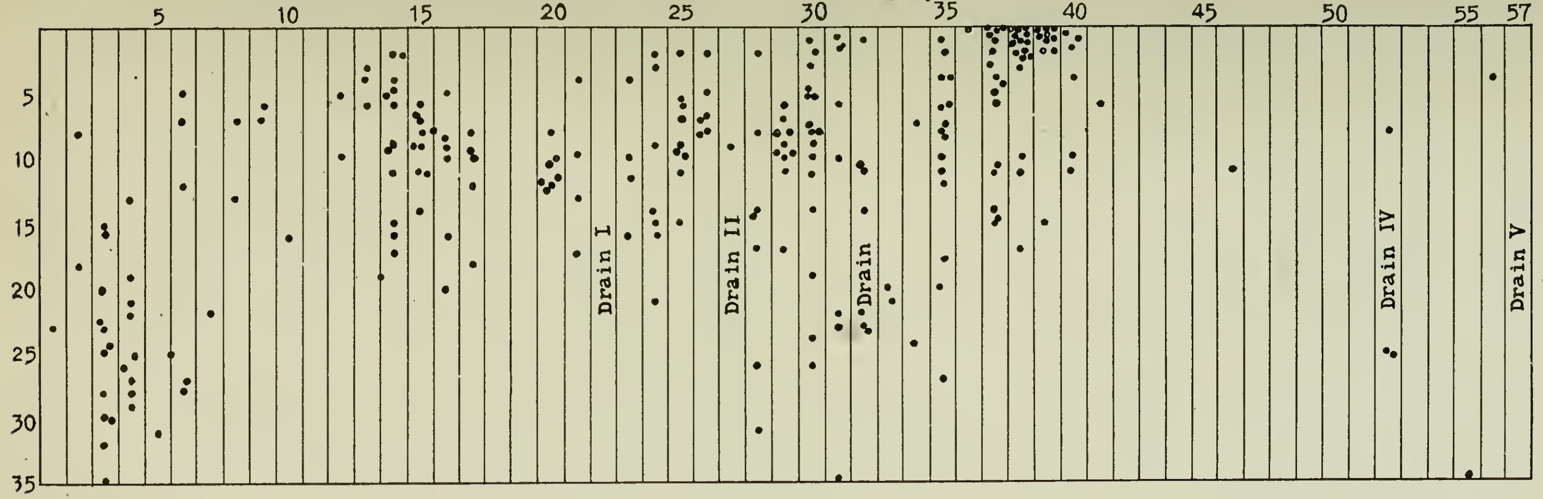


Chart 1 gives the results of these seven surveys. These surveys will be taken to represent the original conditions in the cane field. Chart 2 shows graphically the distribution of stools found in the seven original surveys. This diagram shows that while the pollution was considerably scattered there were definite centers of concentration, probably brought about by the easiness of access and proximity to the barracks. A number of points in our findings indicated that it was a common thing for a certain individual to visit the same place day after day, which would also tend to concentrate the pollution. This production of pollution centers in the cane was very important, since in this way were produced centers of soil infestation which were visited by the people of the area. It will be noticed from the chart that the great majority of the stools were deposited within fifteen yards of the edge of the cane. This holds for the pollution in rows 1 to 5, as well as elsewhere, since there was a large open space in this corner of the field, so that the edge of the cane was from five to twenty yards from the edge of the field. In the early surveys it will be noticed that numbers of stools were deposited from twenty to thirty-five yards from the edge of the cane. This was made possible by the fact that the cane was far from full grown at the time of these surveys, and it was easy to go down the rows (see Figs. 2 and 3).

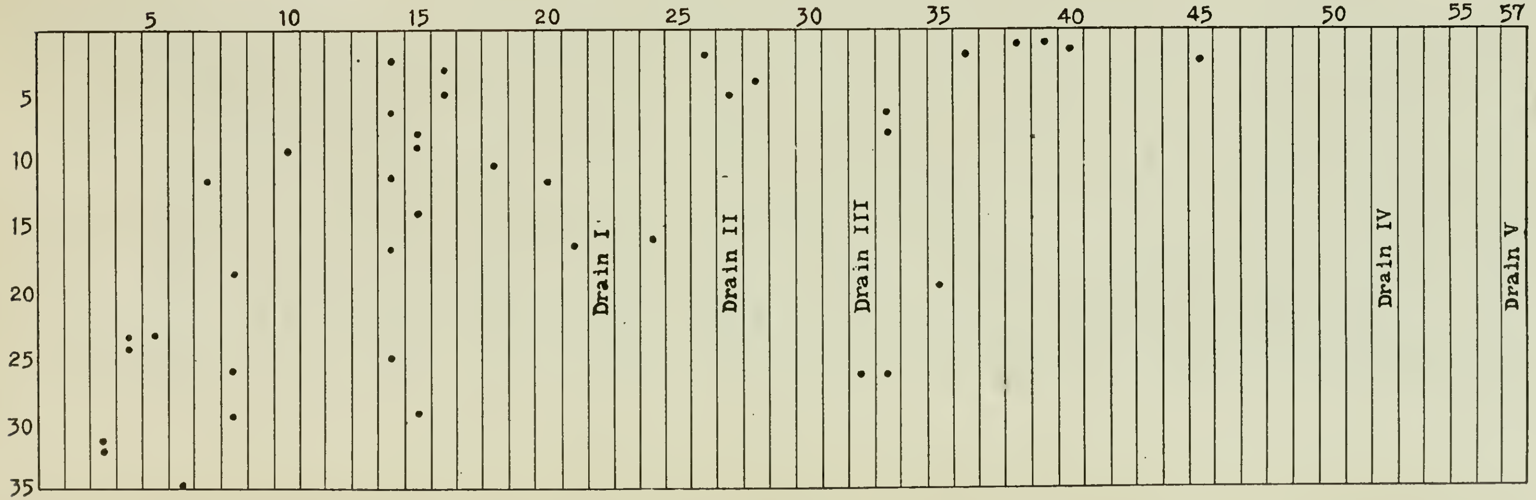
Later the growth of the cane made access to many of the rows practically impossible. This factor would tend to increase the concentration of pollution and therefore the danger of infestation. The position of the centers of pollution, as shown on Chart 2, can be readily explained. Rows 13, 14, 15 and 16 were fairly wide, giving easy access from barrack 13, and offered good shelter. The same condition with relation to other barracks held good in such rows as 20, 25, 26 and 35. The pollution in rows 29, 30 and 31 was concentrated in an open space protected by a fringe of cane at the edge. This place was very heavily polluted early in the work, but later the pollution shifted away from here, as will be described later. One

CHART 1. This chart shows the original seven pollution surveys made in the cane field area before the introduction of the educational campaign. The figures to the left indicate the number of yards from the edge of the field. The rows are shown by parallel lines, and the numbers of the rows are given along the top and bottom of the page. The position of the drains is shown by two parallel dotted lines, and the numbers are given in survey 1. The dots indicate the position of fresh human stools found on the morning of the surveys. All the surveys were made from 9 to 11 A. M.

A SURVEYS 1-7



B. SURVEYS 8-14



explanation for this was that while at the beginning of our study very little grass was present in this area, later it grew up to a heavy growth, which might have easily become objectionable to those visiting the place. The pollution center in rows 37 to 39 was an open space at the edge, where there was no cover. While both East Indians and negroes were known to visit the cane, it is to the former that the pollution of the cane was chiefly due. Besides the pollution shown on the diagram there was another center found in row 55, fifteen yards from the edge of the cane. A stool and evidence of previous pollution was found at this spot on June 22, but on no later date were other stools found, although the place was observed at every subsequent visit to the area.

A possible source of error in the pollution surveys was the difficulty in determining how old the traces of stool were. The plan of the survey was to count only recent stools in order that only a single day's pollution would be recorded in a single survey. Usually the stools disappeared with remarkable rapidity, but there was some delay in dry weather. Usually after twenty-four hours the stool would be leveled to the ground. In fact the loose soil of the cane field, the heavy rains and the intense activity of the insects made it possible for a very large amount of pollution to be absorbed into the soil in a short time.

Since it was possible to accurately measure soil pollution in the area of the cane just described, it was decided to continue the pollution surveys during and after an intensive hookworm campaign to test the results obtained.

Conditions which might influence soil pollution in the cane began to act as soon as we started our work in this area on June 22. It is possible that our early visits may have had an influence in keeping the people from visiting the cane field, but the pollution surveys, up to July 18th, show very little reduction in the daily deposit of stools. At the time of our second visit to this area on June 24, it was found that both the old latrines (Nos. 1 and 2) had been cleaned and moved,

CHART 2. This chart gives a summary of the surveys made in the cane field area. (*a*) Summary of surveys 1–7 (see Chart 1), which were made before the introduction of latrines and the control measures reduced the amount of soil pollution. (*b*) Summary of surveys 8–14 which were begun on July 22 after the introduction of latrines and educational work had reduced soil pollution and were continued at intervals until August 31 (see Chart 3). For further details see description of Chart 1.

and by about June 25 were put in fair shape for use. Soon after this, work was begun on the new latrines, and by July 9th four new units, latrine No. 5, were completed and in use. By July 15, the second four new units (latrine No. 4) were completed and soon were brought into use. By July 20 the last of the new latrines (latrine No. 3) was completed, and 14 units in five separate latrines were in use (see map). These latrines were in use up to the end of our work, and although they were at times allowed to become dirty, they represented a great improvement over the previous condition. That the bringing of these latrines into use did not at once reduce appreciably the pollution in the cane is shown by the surveys of July 8 and July 18, in which there was shown very little if any reduction.

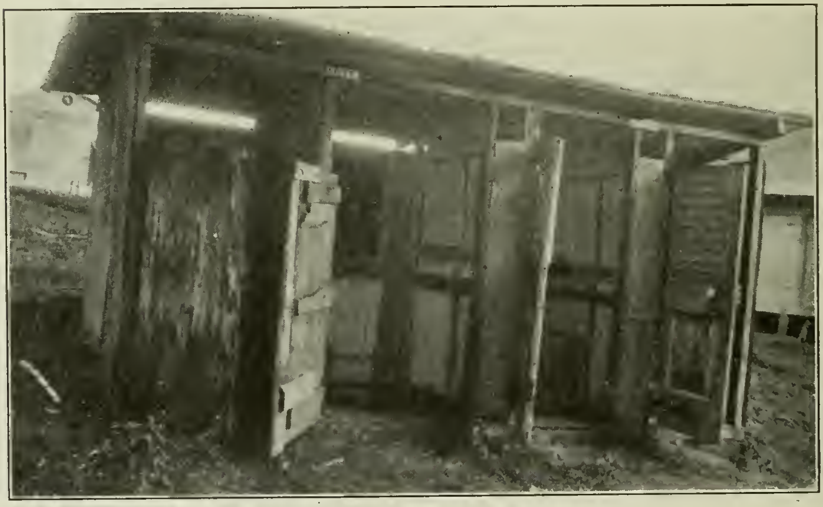


FIG. 10. Type of latrines built by the manager of the estate during the course of the work. Those of the seat type were reserved for the negroes, and of the squat type for the East Indians.

The type of new latrines built in this area is shown in Fig. 10. Special stalls were reserved for East Indians and negroes, with signs above the doors. The units reserved for the Indians had squatting platforms instead of seats. The pits under these latrines were about six feet deep and three feet wide. They were surrounded by banks of earth, outside of which were drains to divert the flow of surface water from the pits.

To supplement the introduction of the latrines educational work was instituted. On July 13 a trained East Indian was sent into the area. On this day and several days following he held demonstrations. In these demonstrations an attempt was made to acquaint the people with the effects of hookworm disease, its cause and methods of control. Especial emphasis was laid on the relation of soil pollution to the spread of the disease. To aid in the demonstration this worker was provided with an album of photographs and a series of colored charts, showing in an oriental allegory the main facts about hookworm disease. By arranging the demonstration in the afternoon, after the people had returned from the field, the great majority were reached. After this first series of demonstrations nothing more was done until Tuesday evening, July 19, when one of us (Payne) gave an illustrated lecture in the open air. This lecture was divided into two parts, both illustrated by the lantern. The first part gave a brief outline of the cause and method of control of hookworm disease, and the second showed by a series of cases from Trinidad the effects of the disease and the results of treatment. The people were informed of the lecture by the field assistant, and an audience of about 250 attended, including most of the people living in the area being investigated. One of the chief points made in this early educational work was that to control hookworm disease cooperation was necessary, that the estate manager was building the latrines, that the doctors would give the medicine, and that the part of the residents of the barracks was to use the latrines and not to pollute the soil. The point was constantly urged that treatments could not start until there was a reduction of soil pollution. The effect of this educational campaign is shown clearly in the striking reduction of soil pollution in the cane that was found in the survey of July 22, when only eight stools were found, as compared with the twenty-seven on July 18. Later surveys, as seen in Chart 3, continued to show this greatly reduced pollution, up to the time of closing the work. The latrines during this period came into constant use and became quite dirty. They were cleaned at times, but no regular system of cleaning was worked out. While this did not influence the present study it must be pointed out that unless latrines are kept reasonably clean, it is difficult to hold the ground gained by the educational work in the reduction of soil pollution. Treatment started July 22 and continued for several weeks. From this time until the end of our work there was at the barracks daily a nurse, who, in addition to administering treatment, instructed the people and answered questions. This nurse on Sunday, August 14, gave a series of microscopical demonstrations, showing adult hookworms, eggs and infective larvae. These demonstrations were accompanied by further explanations on the methods of transmission and control of hookworm disease. During all the work, there was a most hearty and interested cooperation by the manager of the estate. The educational campaign just described is the usual educational campaign of the Trinidad Ankylostomiasis Commission in an East Indian district.

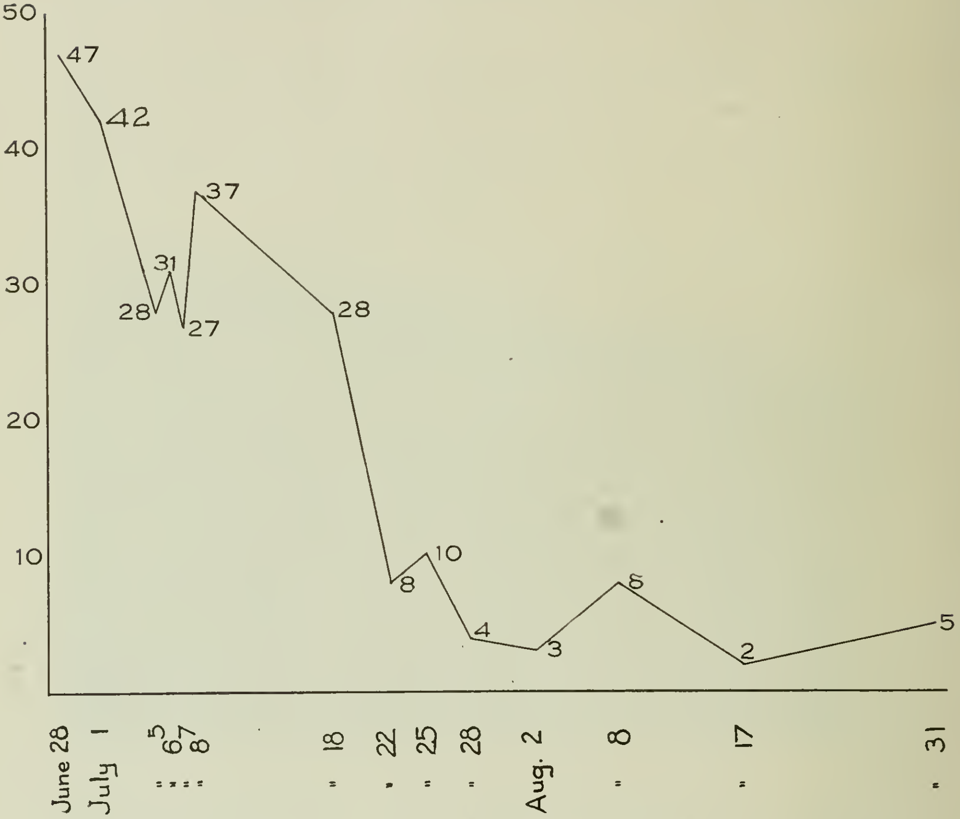


Chart 3. This chart is a curve to show the reduction of soil pollution in the cane field area, produced by the introduction of latrines and the educational work. The abscissa gives the length of time of the experiment, the units being one day each. The days on which the surveys were made are given. Along the ordinate is indicated the number of stools found.

No attempt was made to make the methods more intensive than those usually used. Therefore, the striking results in the reduction of soil pollution obtained illustrated (see Chart 3) what can be done by the usual methods, and is in reality merely a test of the effect of the routine educational campaign on soil pollution.

RESULTS OF EXAMINATION OF SOIL SAMPLES FOR HOOKWORM LARVAE.

The original purpose of the examination for hookworm larvae of soil samples from the sugar cane area was to determine the distribution of soil infestation, with the idea of locating the exact sources of the infestation of the people of the area. Incidentally data were obtained relating to the activities of infective hookworm larvae in the soil.

Attempts to discover the sources of human infestation usually consist of (1) a more or less accurate study of soil pollution, (2) an analysis of the habits of the people with regard to contacts with places that have been polluted, and (3) observations on the clinical side, especially in regard to ground itch. Although information from these sources is important, all such studies must be incomplete and often misleading, unless they include an investigation of the distribution of infective hookworm larvae in the soil. Up to the time of the researches of Baermann (1917*b*), the study of the distribution of infective hookworm larvae in the soil was practically impossible, because no apparatus had been devised by which they could be effectively isolated even from comparatively small quantities of soil. This difficulty has now been overcome (see Baermann 1917*b*, and Cort, Ackert et al., 1922), and it is now possible to locate the hookworm larvae even in comparatively large samples of soil. Baermann (1917*a*) in his extensive epidemiologic studies in Sumatra utilized the apparatus for the isolation of infective hookworm larvae from soil in the study of the sources of human infestation under a variety of different conditions. Most striking were his studies by this method of the sources of reinfestation in places where intensive control campaigns had been carried through, but where it had been impossible to eradicate the disease. His finding in this study of unsuspected and very important centers of soil infestation, in the wet spots inside of the houses (Baermann 1917*a*), shows how impossible it is to truly estimate the sources of human infestation without examining the soil for infective larvae.

The method, which we used to determine the location of hookworm larvae in the soil, was similar to that used by Baermann in his investigations. Samples of soil were brought in from the field in pint tin cans, set up in the isolation apparatus, and on the following morning the infective hookworm larvae obtained were counted. The technic of using the isolation apparatus was described in an earlier number of this series (Cort, Ackert et al., 1922). For recording the soil samples from the field special blanks were prepared as shown below.

SOIL SAMPLE RECORD.

No.

District *House No.* *Area*
Date *Time* *Rainfall for prior 48 hours*
Result of Examination: Hookworm Larvae *Free Nematodes*
Location

Soil Kind
Moisture Content: *Saturated* *Wet*
Moist *Slightly Moist* *Dry*
Shade
No Shade
Light Shade
Deep Shade
Vegetation

Possible Sources of Pollution

Danger to Inhabitants

Remarks:

These were given serial numbers to correspond with numbers placed with the soil in the tin containers and the necessary information filled in at the time the samples were taken. The locations from which the samples were taken were determined by the particular information desired. Since experiments by Augustine (1922*b*) and others of the Trinidad expedition had shown that as long as the soil is moist, the infective hookworm larvae were almost exclusively found at or very near the surface, only the surface layer was included in the sample. Usually the dirt for a single sample was obtained by scraping the surface of a small area or from several small spots over a somewhat larger area.

In all 233 soil samples were examined from the area under consideration. It will be impossible to include in this account all the data from the blanks which were filled out. A complete list of the samples and a summary of the results of the examinations is given in Tables 1 to 7.

Since the soil of this area was a clay loam, varying only in the percentage of clay present, the type of soil in all the samples was practically the same. Since the examinations were made during the wet season the soil was always at least moist and usually wet or saturated. The conditions of shade and vegetation, at the places where the samples were taken, seemed to be a factor in the presence of larvae. It was impossible to include these data for each sample, and they will be discussed for general groups of samples. Also the " possible sources of pollution " and " danger to inhabitants " need not be taken up for each individual sample. In practically every sample examined there were found free living nematodes varying in numbers from a few to several thousand. In addition there were found several different types of sheathed nematode larvae, which were thought to belong to Strongylidae of the cattle and goats on the area. To aid in the identification of the infective hookworm larvae, a few drops of 5 per cent. formalin would be mixed on the slide; this killed the free-living nematodes and made it much easier to study the preparations. A more detailed account of the technic of examining samples is given in an earlier number of this series (Cort, Ackert et al., 1922).

It will be noted from the summary (Table 1) that of the samples from the area 159 were taken from the strip of the cane field which was studied, and 74 were taken from places near the houses or in the yards. The map of the area shows graphically the location of the samples taken and the approximate results of the examinations. We will discuss first the samples taken outside the cane field and later the more intensive examinations of the strip of the cane field. As noted previously (Cort, Augustine et al., 1922) a large proportion of the infective hookworm larvae found were unsheathed.

The examinations outside the cane field developed into a hunt for larvae and the soil samples were taken with relation to known polluted places, places where pollution was suspected and places where human

TABLE 1.

Summary of samples from area.

No.	Location.	–	+	Hookworm Larvae Found.		
				Sheathed.	Unsheathed.	Total.
6	Drain 1	3	3	7	1	8
19	Drain 2	6	13	25	42	67
19	Drain 3	4	15	78	367	445
6	Drain 4	5	1	16	19	35
11	Drain 5	4	7	9	134	143
61	Drains 1–5	22	39	135	563	698
8	Rows 2, 3, 4, 6, 8	2	6	59	217	276
14	Rows 14, 15, 16	1	13	495	153	648
5	Rows 20, 23, 25, 26	0	5	93	111	204
31	Rows 29, 30, 31	7	24	172	196	368
21	Rows 35–40	11	10	238	180	418
19	Rows 55	8	11	601	1,052	1,653
98	Between Rows	29	69	1,658	1,909	3,567
21	Around Latrines	9	12	72	51	123
10	At ends of cane paths	4	6	6	14	20
43	Around houses	33	10	26	58	84
74	Total outside cane	46	28	104	123	227
61	Drains 1–5	22	39	135	563	698
98	Between Rows	29	69	1,658	1,909	3,567
159	Total from cane	51	108	1,793	2,472	4,265
74	Total outside cane	46	28	104	123	227
233	Total area C	97	136	1,897	2,595	4,492

contact with the soil was frequent. In this connection samples were also taken from around the latrines to test their importance as possible sources of infestation. The results of the examination of these samples are recorded in Tables 2, 3, 4 and 5, and the location from which they were taken is shown on the map of the area. The remarkable thing about these results was the small number of larvae isolated. The findings from the various types of situations will be discussed in some detail.

TABLE 2.

Wet spots under the galleries and near barracks.

No.	Date.	Location.	Larvae Found.		
			Sheathed.	Unsheathed.	Total.
208	July 25	B.2. W. side N. of steps under floor.	..	..	0
209	"	B.2. At sides of drain N. of house in front of second door from E......	..	..	0
210	"	B.3. S.W. cor. house around water barrel..........................	..	..	0
211	"	B.3. ditto.........................	..	..	0
212	"	B.4. Next to S.E. cor. of house....	..	..	0
213	"	B.5. At S.E. cor. of house..........	..	..	0
214	"	B.6. Just out from S.E. cor. of house	..	..	0
215	"	B.7. Around boards directly W. of house..........................	..	..	0
216	"	B.8. Directly W. of house..........	..	.	0
217	"	B.9. Around bridge directly W. of house..........................	..	..	0
218	"	B.10. W. of middle door in drain around boards..................	..	..	0
118	July 8	B.11. S.E. cor. gallery, at inner end of bridge of iron pipes...............	..	..	0
119	"	B.11. S. edge of gallery, at inner end of pipes across drain.............	..	..	0
120	"	B.11. 2 yards E. of N.W. corner of gallery inside gallery..............	..	1	1
121	"	B.11. 2 yards W. of S.W. corner of gallery in path....................	..	..	0
122	"	B.11. 4 yards S.W. from 121 along path..........................	..	..	0
123	"	B.11. 5 yards S.W. 122, where path from house 11 joins path from 12...	..	..	0
124	"	Just at edge of gallery of cowshed where path crosses drain..........	..	..	0
125	"	B.12. S.E. cor. of gallery, just outside, across drain.................	..	..	0
126	"	B.12. At E. end of gallery next to house........................	1	..	1
127	"	B.12. Along outer edge of gallery next to drain.........................	..	..	0
128	"	B.12. At front of gallery in front of middle door, at inner end of bridge.	..	..	0
129	"	B.12. In gallery E. of 1st steps toward W. against house.................	..	..	0
130	"	B.12. At inner end of ditch just E. of bridge across drain at W. end......	..	..	0
131	"	B.13. Just W. of S.W. corner past house..........................	..	..	0

No.	Date.	Location.	Larvae Found.		
			Sheathed.	Unsheathed.	Total.
132	July 8	B.13. 3 yds. W. of house, where path from house crosses drain..........	..	..	0
133	"	B.13. At edge of gallery just W. of W. bridge.........................	..	..	0
134	"	B.13. At edge of gallery, opposite two W. water barrels, inner end of bridge	..	..	0
135	"	B.13. At inner edge of drain along gallery, W. of water barrels........	..	..	0

Stimulated by the findings of Baermann (1917*a*) in regard to soil infestation within and near houses a series of samples was taken near the barracks. Since all the barracks on our area had wooden floors, there were no conditions exactly comparable to the wet spots which Baermann found inside the "pondoks" in Sumatra. Similar to these places, however, were wet spots under the galleries, or just outside the shelter of the houses, often near water barrels where the people washed themselves or their utensils. Most of these places were shaded for all or part of the day, were constantly wet and often visited by the people. They were also alike in being without grass and in the soil being very solidly packed. No visible signs of pollution were found at any of these spots. Soil samples, totaling twenty-nine in number, were taken from all the places of this type in the area. The results of the examination of these samples are given in Table 2. It will be seen that there were only two positives, with one larva each. An analysis of Baermann's findings in regard to soil infestation of the wet spots indicates very strongly that situations of the type just described would under conditions in Sumatra have had a heavy soil infestation. In trying to explain the lack of soil infestation of these wet spots in our area several possibilities suggest themselves. In considering environmental conditions it would appear that there was plenty of moisture in these situations, and that many of them were continually shaded. The only unfavorable factors which suggest themselves are the type of soil which was a clay loam, rather solidly packed, and the lack of any vegetation on the soil. Since there was no evidence of pollution found at these spots it is of course possible that hookworm eggs and larvae were not brought to them in sufficient quantities to produce soil infestation. In one respect conditions differed from those found by Baermann in Sumatra, in that while the people there were accustomed to wash themselves after defecation, at the wet spots within or near the houses, in Trinidad it was the custom of the East Indians to carry a bottle of water to the place of defecation for this purpose. However, in view of the widespread soil pollution in this area, even close to the houses, and of the fact that the people washed their feet at these places, it is hard to believe that fecal material and larvae would not be carried there.

TABLE 3.

Polluted places near barrack 13.

No.	Date.	Location.	Larvae found.		
			Sheathed.	Unsheathed.	Total.
147	July 11	B.13. Mound W. of gallery, 2 yds. W. of gallery support	..	..	0
148	"	B.13. 2 yds. W. of 147, on mound	..	4	4
149	"	B.13. 1 yd. S. of 148, on mound	..	5	5
150	"	B.13. 1 yd. S.W. of 149	..	14	14
151	"	B.13. 3 yds. S. of middle of barracks, in path	..	..	0
152	"	B.13. 5 yds. S. on path from 151	2	2	4
153	"	B.13. In polluted area in front of barracks	3	1	4
219	July 25	B.13. In polluted area in front of barracks	..	..	0
220	"	B.13. In polluted area in front of barracks	..	..	0
221	"	B.13. In polluted area in front of barracks	20	27	47
222	"	B.13. In polluted area in front of barracks	..	3	3
223	"	B.13. In polluted area in front of barracks	..	..	0
224	"	B.13. In polluted area in front of barracks	..	1	1
225	"	B.13. In polluted area in front of barracks	..	..	0

The results of the examinations of a series of samples from a somewhat different type of location are shown in Table 3. These samples were taken near barrack 13, at places where there was a considerable amount of soil pollution. Samples 147 to 150 were taken on a mound, just west of barrack 13. This mound was a very heavily polluted spot, evidently a favorite place with the children. It received morning shade and was covered with sparse grass and debris from the house. With the amount of pollution here the finding of three out of four positive samples is not surprising. The rest of the samples in Table 3 were taken from an area in front of barrack 13 (see map). This area was planted to tannia, grown up thickly with weeds, and partly shaded by a large cedar tree. It was constantly polluted, especially by the children. The findings from this place show a considerable amount of scattered soil infestation, but certainly not commensurate with the amount of pollution. In fact in both of the areas from which the samples in Table 3 were taken, we were very much surprised at the small numbers of larvae found, as compared with the grossness of the pollution. It is possible that here too the conditions were not the best for the development of the larvae, although their small numbers might be explained by the fact that most of the pollution near the house was by children, who were not as heavily infested as the adults. The lack of uniform distribution of the larvae over the comparatively small areas near barrack 13 indicates that they do not migrate widely in the soil.

Table 4 gives the results of the examination of samples taken around the latrines. Samples 1 to 9 were taken with regard to the possibility of one of the original latrines being a source of infestation. The condition of this latrine (latrine No. 1) at the time of this examination has been described above. Since the whole inside was smeared with feces, and the whole area in front was polluted both with whole stools and feces tracked from inside, it can be seen that there was a very great concentration of soil pollution. In considering samples 1 and 2, taken directly in front of this latrine, it is surprising that more larvae were not found. In fact, the place where sample 1 was taken was almost constantly smeared with feces, and the fact that only two larvae were found indicates that the situation was unfavorable for development. The soil here was almost pure clay, closely packed and without grass. It was shaded in the morning, but exposed to the direct rays of the afternoon sun. Sample 2 was taken from the same situation, only one foot from 1, but differed in being taken around the roots of a clump of grass. A comparison of the findings from these two samples shows that in clay soil, especially

TABLE 4.

Near the latrines.

No.	Date.	Location.	Larvae found.		
			Sheathed.	Unsheathed.	Total.
1	June 22	Latrine I.* 1 ft. in front of door, ground bare.........	2	..	2
2	"	" I. 1 ft. south of 1, in grass.	19	32	51
3	"	" I. Along path 1 yd. from 2	14	3	17
4	"	" I. " " 3 yds. " 2	2	1	3
5	"	" I. " " 7 " " 2	3	1	4
6	"	" I. " " 10 " " 2	8	3	11
7	"	" I. " " 15 " " 2	2	4	6
8	"	" I. Directly back of middle.	7	1	8
9	"	" I. 1 ft. directly back of 8 ..	2	4	6
309	Aug. 2	" I. Along front............	..	..	0
310	"	" I. In path to south.......	..	..	0
311	"	" I. Along path to south from 1 yd. to 6 yds........	..	..	0
302	"	" II. Along front............	..	..	0
303	"	" II. Along path to west of 302	2	..	2
300	"	" III. All along front.........	9	1	10
301	"	" III. Along path to north....	..	..	0
304	"	" IV. Along front............	..	..	0
305	"	" IV. On path to north.......	2	1	3
306	"	" IV. Along path to south....	..	..	0
307	"	" V. Along front............	..	..	0
308	"	" V. Along path to south....	..	..	0

when packed hard, the presence of grass which gives protection and loosens the soil will make the situation favorable for the development of hookworm larvae. Samples 3 to 7 are of interest, since they show the possibility of the spread of soil infestation along a path. The path was covered with a dense grass and so conditions were favorable for development of larvae. It seems certain that this latrine under the conditions which existed prior to June 22 was a source of infestation with hookworms to those visiting it.

Samples 300 to 311 were taken at the most favorable places for pollution and human contact in connection with the new latrines which were installed in this area, after they had had time to become dirty. There was considerable fouling of the places from which these samples were taken before July 22 when the first treatments were instituted, so that there was opportunity for considerable soil infestation, if the conditions had been favorable. The only shade which the situations had was from the latrines, which was only effective for part of the day. Samples 300, 304, 306, 307 and 310 were taken from situations where there was no grass, while the others were protected by grass of various densities. Of the three positives only one (300) was unprotected by grass. This indicates that the larvae can occasionally develop in situations where there is very little protection.

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* Samples 1 to 9 were taken from original location, marked old pit on map.

In this whole series from around the latrines the small numbers of larvae found again indicate how difficult it is for soil infestation to develop, even with gross pollution by heavily infested individuals, when the soil is a hard-packed clay or clay loam. These findings in regard to latrines suggest that as a precaution against soil infestation the area immediately around a latrine should, if possible, be built up with a solid clay or cement platform and kept clear of grass.

TABLE 5.

At ends of paths into the cane.

No.	Date.	Location.	Larvae found.		
			Sheathed.	Unsheathed.	Total.
136	July 11	54th row. At edge of bank where path enters cane...........	..	..	0
137	"	" " 12 ft. directly east along path from 136.........	..	..	0
138	"	39th " At edge of bank on path into cane.............	3	..	3
139	"	" " 1 yd. up path from 138..	..	..	0
140	"	35th " At bottom of bank on path into cane.............	1	1	2
141	"	" " On path at top of bank...	..	..	0
143	"	33d " On path two yds. east of edge of cane...........	..	2	2
144	"	2d " On path at edge of bank at edge of cane...........	1	7	8
145	"	1½ yds. south of 144 along path.....	..	2	2
146	"	3 yds. south of 144 along path.......	1	2	3

Table 5 gives the results of examinations of samples taken at or near the ends of the paths leading into the cane field. Since we had no evidence of pollution in these paths it is probable that the results of the examinations illustrate the possibility of establishing soil infestation by the transportation of hookworm eggs and larvae on human feet. All these paths were covered with a dense growth of grass, but were otherwise unshaded. The possibility of transportation of hookworm larvae on the feet is brought out by a series of examinations of the mud from the feet of individuals visiting the cane field, which gave hookworm larvae in considerable numbers (see Augustine 1922*a*, p. 169).

To make an intensive study of the distribution of hookworm larvae in the cane field to the west of the barracks, a series of 159 soil samples was examined from the heavily polluted area extending for over a hundred yards along the west side of the barracks and for thirty-five yards into the field (see map). A detailed study of the soil pollution in this area was recorded earlier in this paper and should be considered in connection with the results of the examination of the soil samples. The samples were taken, except in the drains, in direct relation to the centers of pollution, and a comparison of Chart 2*a* with the map of the edge of the cane gives graphically an idea of the relation between soil pollution and soil infestation in this area. Soil samples were taken from three general types of situations, viz., (1) between definite rows of cane, (2) in open spaces at the edge or a short distance back into the cane, and (3) from the bottoms of the drains. The summary of the results of these examinations (Table 1) shows a very heavy soil infestation, for out of the 159 samples examined only 51 were negative for infective hookworm larvae, and 108 were positive. From these 108 positives, 4,265 infective hookworm larvae were isolated, of which 1,793 were sheathed and 2,472 were without their protective sheaths. The significance of finding such a large proportion of the infective larvae without their protective sheath has been discussed in another connection (Cort, Augustine et al., 1922). From this intensive study of soil infestation in this strip of cane field data were obtained on the relation between soil pollution and soil infestation and on the activities of infective hookworm larvae in the soil. We will discuss in some detail the results of the findings. For convenience the findings from between the rows will be discussed separately from those in the drains.

All the samples from between the rows were taken with relation to definite centers of soil pollution. The results of the examinations

TABLE 6.

From between rows of cane field strip.

Sample No.	Date taken.	Location.	Hookworm larvae found.		
			No. sheathed.	No. unsheathed.	Total.
191	July 18	R. 2; 32	..	..	0
82	“ 4	R. 3; 16	..	..	0
83	“ 4	R. 3; 21	31	2	33
190	“ 18	R. 3; 33	16	66	82
84	“ 4	R. 4; 30	..	4	4
85	“ 4	R. 4; 35	..	8	8
192	“ 18	R. 6; 12	12	136	148
193	“ 18	R. 8; 10	..	1	1
75	“ 1	R. 14; 3	1	..	1
88	“ 4	R. 14; 3	..	1	1
154	“ 13	R. 14; 4	1	36	37
76	“ 1	R. 14; 6	..	..	0
155	“ 13	R. 14; 7	2	9	11
77	“ 1	R. 14; 9	..	1	1
89	“ 4	R. 14; 11	407	..	407
78	“ 1	R. 14; 12	..	1	1
90	“ 4	R. 15; 5	2	4	6
156	“ 13	R. 15; 6	72	21	93
91	“ 4	R. 15; 7	2	6	8
157	“ 13	R. 15; 9	7	68	75
194	“ 18	R. 15; 13	..	4	4
195	“ 18	R. 16; 7	1	2	3
196	“ 18	R. 20; 10	18	10	28
197	“ 18	R. 23; 7	8	13	21
199	“ 18	R. 25; 5	2	1	3
198	July 18	R. 25; 10	..	35	35
200	“ 18	R. 26; 7	65	52	117
201	“ 18	R. 28; 3	1	..	1
202	“ 18	R. 28; 5	3	14	17
50	June 29	R. 29; 8	..	..	0
68	July 1	R. 29; 8	1	..	1
203	“ 18	R. 29; 10	35	5	40
158	“ 13	R. 29; 8	2	14	16
16	June 24	R. 30; 1	2	10	12
159	July 13	R. 30; 1	..	2	2
17	June 24	R. 30; 2	5	31	36
18	“ 24	R. 30; 3	..	2	2
19	“ 24	R. 30; 5	8	21	29
160	July 13	R. 30; 6	..	..	0
161	“ 13	R. 30: 7	..	..	0
20	June 24	R. 30; 7	2	24	26
162	July 13	R. 30; 10	..	8	8
21	June 24	R. 30; 10	7	13	20

TABLE 6—*Continued.*

Sample No.	Date taken.	Location.	Hookworm larvae found.		
			No. sheathed.	No. unsheathed.	Total.
22	" 24	R. 30; 13	..	7	7
31	" 27	R. 30; 16	15	2	17
163	July 13	R. 30; 18	..	1	1
23	June 24	R. 30; 19	6	13	19
164	July 13	R. 30; 22	29	4	33
32	June 27	R. 30; 22	16	7	23
33	" 27	R. 30; 25	18	7	25
165	July 13	R. 30; 27	..	1	1
34	June 27	R. 30; 28	10	5	15
46	June 29	R. 30; 33	..	..	0
47	" 29	R. 30; 38	..	..	0
48	" 29	R. 30; 43	..	..	0
49	" 29	R. 30; 48	..	..	0
166	July 13	R. 31; 5	5	..	5
51	June 29	R. 31; 10	7	5	12
167	July 13	R. 35; 4	20	..	20
168	" 13	R. 35; 9	2	15	17
169	" 13	R. 37; 1	2	..	2
102	" 6	R. 37; 2	13	..	13
181	" 13	R. 37; 3	..	4	4
79	" 1	R. 37; 3	..	..	0
103	" 6	R. 37; 4	36	6	42
80	" 1	R. 37; 6	..	..	0
81	" 1	R. 37; 9	..	..	0
204	" 18	R. 37; 12	161	150	311
170	" 13	R. 38; 1	..	..	0
104	" 1	R. 38; 1	..	..	0
182	July 13	R. 38; 3	..	..	0
105	" 6	R. 38; 4	..	..	0
205	" 18	R. 38; 8	..	1	1
171	" 13	R. 39; 1	..	..	0
106	" 6	R. 39; 2	3	1	4
183	" 13	R. 39; 3	..	..	0
107	" 6	R. 39; 4	1	3	4
206	" 18	R. 40; 5	..	..	0
207	" 18	R. 40; 9	..	..	0
57	June 29	R. 55; 1	..	..	0
58	June 29	R. 55; 3	..	..	0
59	" 29	R. 55; 7	..	..	0
60	" 29	R. 55; 10	..	..	0
184	July 13	R. 55; 11	..	..	0
185	" 13	R. 55; 13	..	..	0
11	June 22	R. 55; 14½	17	126	143

TABLE 6—*Continued.*

Sample No.	Date taken.	Location.	Hookworm larvae found.		
			No. sheathed.	No. unsheathed.	Total.
65	July 1	R. 55; 14½	208*	209*	417
10	June 22	R. 55; 15	312*	625*	937
64	July 1	R. 55; 15	18	35	53
186	" 13	R. 55; 15	..	9	9
12	June 22	R. 55; 15[1]	20	17	37
67	July 1	R. 55; 15[2]	14	10	24
13	June 22	R. 55; 16	7	10	17
66	July 1	R. 55; 16	..	1	1
14	June 22	R. 55; 17	5	7	12
62	" 29	R. 55; 17	..	..	0
15	" 22	R. 55; 19	..	3	3
63	" 29	R. 55; 20	..	..	0

* Approximate count and estimate.

[1] 1½ ft. north from 10.

[2] 1 yd. south from 10.

of these samples are given in Table 6. A study of this table in connection with the map of this area will give a pretty clear idea of the distribution of soil infestation in the cane. Most striking perhaps is the very large number of larvae isolated from this area and their concentration at places of intense pollution.

That even in the cane area soil infestation is definitely limited by the effect of the environment on the development of the larvae is shown by the comparison of soil pollution with soil infestation. A very striking example of this point is the condition at the open space, at the edge of the cane, at rows 37, 38 and 39. This area extended about four yards back from the edge and during the early examination was practically without vegetation. The ground at this place was packed hard and there was a high proportion of clay in the soil. This place, with an area of about twenty-four square yards, as can be seen from Chart 2*a*, was the most heavily polluted spot in the cane field, for in the seven original pollution surveys a total of thirty-two stools were recorded. Yet the examination of Table 6 shows that, as compared with other pollution centers, it had a very light soil infestation. A total of thirteen soil samples, on three different days, July 1, 6 and 13, were taken from this place, viz., samples 169, 102, 181, 79, 103, 170, 104, 182, 105, 171, 106, 183, 107. Seven of these were negative and six positive. Only one of the positives (No. 103) had more than a few larvae in it, which might be explained by the fact that this sample was taken from the bottom of a shallow hole which was somewhat protected and received drainage from around. The only explanation we are able to suggest for this very small soil infestation, from a very heavy soil pollution by individuals infested with hookworms, is the hard packed character of the soil and the open unprotected character of this place, where there was no shade and practically no grass or weeds. A comparison of the soil infestation of this place with that of other less heavily polluted spots in the cane, which were protected, shows clearly the importance of shade and vegetation in producing conditions favorable for the development of hookworm larvae. Such a finding illustrates how impossible it is to judge soil infestation or the sources of human infestation by the distribution of soil pollution alone, since not pollution, but pollution plus proper conditions for development, make soil infestation possible.

The findings from the soil samples in the cane field give some significant data on the question of the migrations of the infective hookworm larvae from the place of their development. The soil of the cane is a loose clay loam, containing a considerable proportion of humus, and so light that it would appear to give a favorable medium for the migration of any living organism found in it. The ordinary conception is that infective hookworm larvae are able to migrate actively to an appreciable distance from their place of development and infest the soil over a considerable area. If this were true we would have expected to find a fairly even distribution of the larvae over large portions of the area examined. Instead of this the results show a very definite localization of soil infestation at the places of soil pollution. In a number of cases of two samples taken close together one gave a large number of larvae and the other was negative or contained but few larvae. Several examples from Table 6 will serve to illustrate this point. In row 14 samples 77, 89 and 78 were taken from the center of the row within a radius of three yards. From No. 89, 407 infective hookworm larvae were isolated, while the other two contained only one each. Further examples of the same thing can be obtained from the records of the examination of soil samples from rows 29, 30 and 31. In these three rows there was an area of heavy soil pollution especially in an open space from about two yards to ten yards into the cane. Yet within this area of general soil pollution and soil infestation certain samples taken were found to be negative or almost so. This is strikingly illustrated by samples 50, 68 and 18. Perhaps the best example from this area of failure to migrate is shown in the results of the series of samples taken in row 55. In this row, on June 22, 937 hookworm larvae were isolated from a pint of soil taken from a pollution spot 15 yards into the cane, and on July 1, 417 were found within the same pollution center. How definitely localized this center of soil infestation was is shown by the results of the examination of samples taken on both the above dates near the center. Samples 12, 64, 67, 13, 66, 14 and 62, taken from row 55, illustrate this point, since they show almost no general migration from this center. These specific instances, coupled with the general localized distribution of soil infestation in the cane, show that under the conditions in this strip of cane where everything appeared favorable for migrations, the infective hookworm larvae stayed close to the point of development. There is some evidence that they were carried about by water, but on the whole soil infestation was very much localized. This finding was supported by a series of experiments by Augustine (1922*a*) which showed that under a variety of conditions infective hookworm larvae do not migrate to any appreciable distance.

Certain data were obtained from examination of the soil samples from the strip of cane listed above, which indicated that infective hookworm larvae die out rather quickly in the soil. While the numbers of larvae found in the samples were quite large it struck us early in the work that if the infective hookworm larvae could live for a year or more in the soil, that with the gross daily pollution of this field, by individuals heavily infested with hookworm, the numbers of larvae present at these polluted spots would be exceedingly large.

Data from two particular centers of soil pollution also support this view. For some reason in June and early July there was an appreciable shifting of soil pollution away from the area in the cane, including rows 29, 30 and 31. This has been noted earlier in the section on soil pollution. Our records show that this reduction in pollution was quickly reflected in a reduction in numbers of larvae isolated from samples taken from this area. This is brought out by a comparison of the samples taken from row 30 on June 24 and 27 with the series taken from the same row on July 13. The twelve samples taken at the earlier dates gave 100 per cent. positives with a total of 213 larvae, or 19.2 larvae per sample. The seven samples taken

TABLE 7.

From drains of cane field strip.

Sample No.	Date taken.	Location.	Hookworm larvae found.		
			No. sheathed.	No. unsheathed.	Total.
92	July 4	D. 1; 8	..	..	0
93	“ 4	D. 1; 17	4	..	4
94	“ 4	D. 1; 25	..	..	0
95	“ 4	D. 1; 33	3	..	3
100	“ 6	D. 1; 42	..	1	1
101	“ 6	D. 1; 50	..	..	0
24	June 24	D. 2; 1	..	..	0
25	“ 24	D. 2, 6	..	2	2
172	July 13	D. 2; 8	1	1	2
26	June 24	D. 2; 11	..	1	1
27	“ 24	D. 2; 16	..	3	3
173	July 13	D. 2; 17	4	..	4
28	June 24	D 2; 21	2	19	21
174	July 13	D. 2; 25	..	..	0
29	June 24	D. 2; 26	..	6	6
30	“ 24	D. 2; 31	..	..	0
175	July 13	D. 2; 33	..	1	1
35	June 27	D. 2; 33	9	..	9
36	“ 27	D. 2; 42	2	..	2
37	“ 27	D. 2; 50	5	..	5
38	“ 27	D. 2; 55	..	..	0
55	“ 29	D. 2; 67	1	9	10
56	“ 29	D. 2: 75	..	..	0
73	July 1	D. 2, 83	..	..	0
74	“ 1	D. 2; 92	1	..	1
39	June 27	D. 3; 1	3	1	4
176	July 13	D. 3; 5	1	..	1
177	“ 13	D. 3; 8	1	247	248
40	June 27	D. 3; 8	24	13	37
41	“ 27	D. 3; 17	9	4	13
178	July 13	D. 3; 17	3	20	23
42	June 27	D. 3; 25	10	4	14
179	July 13	D. 3; 25	2	..	2
43	June 27	D. 3; 33	11	1	12
180	July 13	D. 3; 33	..	3	3
44	June 27	D. 3; 37	2	..	2
45	“ 27	D. 3; 40	1	3	4
52	“ 29	D. 3; 50	4	50	54
69	July 1	D. 3; 50	6	21	27
53	June 29	D. 3; 58	..	..	0
54	“ 29	D. 3; 67	..	..	0
70	July 1	D. 3; 75	..	..	0
71	“ 1	D. 3; 83	1	..	1

TABLE 7—*Continued.*

Sample No.	Date taken.	Location.	Hookworm larvae found.		
			No. sheathed.	No. unsheathed.	Total.
72	July 1	D. 3; 165	..	..	0
108	" 6	D. 4; 8	16	19	35
109	" 6	D. 4; 17	..	..	0
110	" 6	D. 4; 25	..	..	0
111	" 6	D. 4; 33	..	..	0
112	" 6	D. 4; 42	..	..	0
113	" 6	D. 4; 50	..	..	0
114	" 6	D. 5; 5	1	32	33
187	July 13	D. 5; 5	..	8	8
96	" 4	D. 5; 8	2	85	87
188	" 13	D. 5; 9	..	1	1
115	" 6	D. 5; 12	4	7	11
189	" 13	D. 5; 13	2	..	2
97	" 4	D. 5; 17	..	..	0
98	" 4	D. 5; 25	..	..	0
99	" 4	D. 5; 33	..	1	1
116	" 6	D. 5; 42	..	..	0
117	" 6	D. 5; 50	..	..	0

on July 13 gave five positives with a total of 45 larvae, or an average of 9 larvae per sample. More striking still in this connection are the findings from the samples taken in row 55. A fresh stool and evidences of former pollution were found at a place about fifteen yards from the edge of this row on June 22. Although this place was inspected on each subsequent visit to the area, no further evidence of pollution was found. The results from samples 11, 65, 10 and 64, taken from row 55, show a very intense center of soil infestation present at this place on June 22 and July 1. Sample No. 186, taken on July 13, gave only nine larvae, and subsequent samples from this place soon showed a complete dying out. The sample of July 13 was taken over the whole area of former soil infestation from about 14½ to 15½ yards down this row, and in our estimation gives a fair test of the reduction of soil infestation. Since the last pollution recorded in this spot was on June 22 this record shows an enormous reduction in soil infestation in a period of only about three weeks. These observations on shortness of life of infective hookworm larvae led to the planning and carrying out of a more comprehensive field experiment, to test in this strip of cane the reduction in soil infestation which follows the reduction of soil pollution. The results of this study will be given in a later section.

Table 7 gives the results of the examination of the soil samples from the five drains which drained the portion of the cane field included in our study. Chart 2*a* shows that only a comparatively small amount of pollution was found in these drains. Further, none of the samples from the drains were taken with relation to stools found in the drains, but at regular intervals. This fact and the high percentage of positives, with the comparatively small numbers of larvae found in most of these samples, indicates a uniform distribution all along the drains rather than the massing at definite polluted centers. An exception to this is sample No. 177 which must have been taken near a polluted spot in the drain. The presence of the infective hookworm larvae in the drains can be accounted for in two ways, viz., (1) as being derived from stools deposited in the drains, and (2) as representing larvae which had been washed into the drains from adjacent centers of soil infestation. Undoubtedly they came from both sources. That infective hookworm larvae were in this case spread by washing from their place of development seems certain from (1) the large numbers of larvae in the drains as compared with the small amount of pollution, (2) their uniform distribution, and (3) that they were found at distances down the drains which were beyond any probability of direct pollution (see Table 7, samples 37, 55, 74, 52, 69, 71). The larger numbers of larvae found in the samples from drains 2 and 3 can easily be explained by the greater concentration of soil pollution and soil infestation in the areas which they drained.

A point already noted (Cort, Augustine et al., 1922) is that the percentage of unsheathed larvae was much greater in the samples from the drains than elsewhere. It is possible that this might be related to the activities of the larvae produced by their being washed from their place of development. The washing of the larvae into the drains by the heavy rains would in a field like this be favorable to control, since they were washed from the places of defecation, which the people visited, into the drains where the people ordinarily would not go.

SOURCES OF HUMAN INFESTATION.

The results of the examination of the soil samples indicate the places where the people of this area would be able to acquire hookworm disease. It remains to discuss the habits of the people which would make possible their infestation from these places. The fact that almost none of the people ever wore shoes, plus the sticky character of the soil, would make possible infestation whenever, as was true during almost all of the rainy season, the soil was muddy. In reviewing the findings it would seem that the strip of cane, west of the barracks, was very largely responsible for the prevalence of hookworm disease in this area. This strip of cane was notorious as a ground itch spot, and for this reason it was difficult for the manager to get the laborers to weed it. Yet, as shown above, this place was constantly visited by the people for the purpose of defecation. The rapid disappearance of the stools made possible the concentrated pollution of a spot, day after day, without producing conditions which would tend to keep people away. Especially significant is the concentration of soil pollution and subsequent soil infestation. The visiting of these places must certainly have been an important factor in the infestation of the people. Imagine visiting day after day with bare feet such places as Row 14, 30 or 55, where hundreds of larvae were found in the soil samples. On wet days the soil of the cane was so sticky that it would cling to our shoes in quantity. As noted above, Augustine (1922*a*, p. 169) found numbers of infective hookworm larvae in mud scraped from our shoes after pollution surveys in the cane field. Also, the bare feet of people were observed on a number of occasions to be literally caked with mud after visiting the cane. We saw cases of ground itch which were attributed to the visiting of the cane. and in fact one of our party contracted ground itch and a light infestation with hookworms through a small hole in his shoe.

Aside from this strip of cane, which must have been responsible for the great majority of the cases of hookworm disease, there were few other places regularly visited which would have produced infestation. The original conditions around latrine No. 1, which were described earlier, would place it as a probable source of infestation to those who visited it. The small findings around the other latrines do not suggest that they formed an important source of infestation. Infestation from immediately around the houses or under the galleries was practically ruled out by the failure to find larvae in these locations. Another source of infestation, especially in the children, would be the stools scattered promiscuously in the grass. Since there was little evidence of concentration of pollution or soil infestation in these places it seems probable that infestation from them would be only occasional and not comparable to that in the cane strip. It seems to us, therefore, that the chief cause of the heavy infestation of the people of this area with hookworms was the habit which many of them had of visiting certain spots near the edge of the cane field for the purpose of defecation.

EXPERIMENTAL STUDY OF THE REDUCTION OF SOIL INFESTATION IN THE CANE FIELD.

The data from the original examinations of soil samples, noted above, which indicated a rapid dying out of larvae in the soil, stimulated us to carry out a field experiment along this line. The strip of cane, west of the barracks, which had been studied so intensively for soil pollution and soil infestation, was chosen for this experiment. The plan of the experiment was, first to bring about a decrease in the number of hookworm eggs deposited on this area, and to make examinations of soil samples at intervals to determine the effect in reducing soil infestation. Two methods were used in reducing the deposit of hookworm eggs in this area. The first was the attempt to reduce soil pollution by the introduction of latrines and educational work, the successful results of which are recorded earlier in this paper. The other was by treatment to reduce the mass infestation of the people of this area and consequently to reduce their output of eggs. The reduction of the soil pollution began between July 18 and July 22 and there was never any return to the previous conditions (see Chart 3). The first treatments were started on July 22. There was therefore between July 22 and August 1 an enormous reduction in the numbers of eggs deposited in the strip of cane under consideration.

The next step was to work out some method by which it would be possible to measure the reduction of soil infestation. It was decided to select from the most heavily infested places in the strip of cane thirty-six spots for examination and to compare the findings from samples taken at intervals from these places. Table 8 gives the location of these spots chosen for examination. Each of the places between rows had an area of from two to four square yards and was staked off at the time when the first series of samples was taken. For the experimental places in the drains the stakes were set about three yards apart. In taking a sample from these places small bits of the surface were scraped off at intervals over the whole area. This method of taking samples in connection with the large size of the experimental areas made it certain that the taking of a single sample would not appreciably diminish the soil infestation of the area.

The first series of thirty-six samples from these areas was taken on July 13 and July 18, before the reduction in soil pollution started and before treatments had been instituted. The records of infective larvae from this series show a very intense soil infestation on this date (see Table 8). Of course it is impossible to know the age of the larvae at the various places where the infestation was found. It is perfectly evident that the larvae infesting the sum total of these areas would contain all ages, and only a comparatively small proportion of them would be newly developed at the time of the first examination. Especially would this be true in certain areas such as Rows 29, 30 and 31, on which, as noted earlier, there had been a considerable reduction in pollution prior to July 13. It is therefore evident that the rate of reduction in the numbers of the larvae from this experimental area would be more rapid than in an experiment in which the larvae would all be fresh from cultures at the start.

The second series of examinations, which was made on August 8 (see Table 8), a little less than three weeks after the reduction of pollution, gave most striking results. There was a reduction of about 93 per cent. in the larvae found, and a number of samples were negative. Especially significant is the fact that the findings from the samples from Rows 29, 30 and 31, where a reduction of pollution before July 13 had been noted, were all negative. The records from the examinations made on August 22 showed still further reductions in the numbers of larvae, and those of September 5, made about six weeks after the reduction of soil pollution, showed an almost complete dying out of the larvae, only seven larvae being isolated from the whole thirty-six samples. In other words in less than six weeks this whole strip of cane had by the practical elimination of the deposit of hookworm eggs been changed from a dangerous ground-itch spot to a place where it was difficult to find any infective hookworm larvae in the soil.

An examination of Table 8 shows that there was a more rapid reduction of larvae in the area south of drain 2. An examination of the pollution surveys (Charts 1 and 2) shows that even before the educational work there was a shifting of pollution away from this part of the field, and that in the later surveys most of the stools were deposited north of this line.

TABLE 8.

Data showing reduction of soil infestation in the cane field strip.

No.	Location.	Ex. I. July 13–18.			Ex. II. Aug. 8.			Ex. III. Aug. 22.			Ex. IV. Sept. 5.		
		S.	U.	Tot.	S.	U.	Tot.	S.	U.	Tot.	S.	U.	Tot.
190	Row 3; 33	16	66	82	17	40	57	...	13	13	...	...	0
192	" 6; 12	12	136	148	1	20	21	...	12	12	1	1	2
154	" 14; 4	1	36	37	...	2	2	...	...	0	...	...	0
155	" 14; 7	2	9	11	1	2	3	...	...	0	3	...	3
156	" 15; 6	72	21	93	...	1	1	...	3	3	...	...	0
157	" 15; 9	7	68	75	...	4	4	...	2	2	...	...	0
194	" 15; 13	......	4	4	6	2	8	...	1	1	...	...	0
195	" 16; 7	1	2	3	1	3	4	1	4	5	...	...	0
196	" 20; 10	18	10	28	...	...	0	...	...	0	...	...	0
197	" 23; 7	8	13	21	...	1	1	4	3	7	...	...	0
198	" 25; 10	......	35	35	2	3	5	1	9	10	...	1	1
199	" 25; 5	2	1	3	...	3	3	...	...	0	...	...	0
200	" 26; 7	65	52	117	2	2	4	...	...	0	...	1	1
202	" 28; 5	3	14	17	5	3	8	...	...	0	...	...	0
203	" 28; 10	35	5	40	...	...	0	1	...	1	...	...	0
158	" 29; 8	2	14	16	...	...	0	...	...	0	...	...	0
159	" 30; 1	......	2	2	...	...	0	...	...	0	...	...	0
162	" 30; 10	......	8	8	...	...	0	...	2	2	...	...	0
164	" 30; 22	29	4	33	...	...	0	...	...	0	...	...	0
166	" 31; 5	5	......	5	...	...	0	...	...	0	...	...	0
167	" 35; 4	20	......	20	1	...	1	...	...	0	...	...	0
168	" 35; 9	2	15	17	...	4	4	...	...	0	...	...	0
204	" 37; 12	161	150	311	...	...	3	...	...	0	...	...	0
186	" 55; 15	......	9	9	...	2	2	...	...	0	...	...	0
172	Dr. 2; 8	1	1	2	...	...	0	...	...	0	...	...	0
173	" 2; 17	4	......	4	...	1	1	...	...	0	...	...	0
174	" 2; 25	......	......	0	...	...	0	...	...	0	...	...	0
175	" 2; 33	......	1	1	...	...	0	...	...	0	...	...	0
176	" 3; 5	1	......	1	1	1	2	...	1	1	...	...	0
177	" 3; 8	1	247	248	...	1	1	...	...	0	...	...	0
178	" 3; 17	3	20	23	...	1	1	...	16	16	...	...	0
179	" 3; 25	2	......	2	...	...	0	...	...	0	...	...	0
180	" 3; 33	......	3	3	...	3	3	...	1	1	...	...	0
187	" 5; 5	......	8	8	...	...	0	...	...	0	...	...	0
188	" 5; 9	......	1	1	...	...	0	...	...	0	...	...	0
189	" 5; 13	2	......	2	...	...	0	...	...	0	...	...	0
Total	Drains	14	281	295	1	6	8	...	18	18	...	...	0
Total	In Rows	461	674	1,135	36	92	128	7	49	56	4	3	7
	Total	475	955	1,430	37	98	136	7	67	74	4	3	7

The reduction in the numbers of larvae in the drains is especially significant since they reflect the general soil infestation of the area which they drain. The first series, from drains of the experimental area, was taken on July 13. Already the results from drains 2 and 5 showed some reduction over previous examinations. The results from drain 3 were especially striking. Up to and including July 13 fourteen samples, all positive, had been taken from the first 50 yards along this drain, with the high average of 32 larvae per sample. The rapid and complete reduction in the numbers of larvae from this drain is shown in Table 8. These particular instances and the general and widespread dying out of the infective hookworm larvae from this strip of cane field show that it was only a matter of six weeks after the elimination of soil pollution until the infestation of the soil practically died out.

This evidence on the shortness of life of infective hookworm larvae in the soil under tropical conditions is supported by the findings from another field exeriment, under quite different conditions (Cort and Payne, 1922*b*), and a series of laboratory experiments (Augustine, 1922*c*). So that the conclusion can be drawn that under tropical conditions, where the high temperatures are favorable to great activity of the hookworm larvae, their life in the soil is quite short.

SUMMARY.

1. An intensive epidemiologic study was made of an area with a high incidence of hookworm disease, in a sugar estate in Trinidad, British West Indies, to determine the exact sources of human infestation and to learn the effect on human infestation, soil pollution and soil infestation of a control campaign.

2. Of the 142 people, both East Indians and negroes, examined from the area chosen for study, 117 were found to be infested with hookworms.

3. A series of three treatments greatly decreased both the number of positives and the total mass infestation of these people.

4. Soil pollution was widespread and very gross in this area, being especially concentrated at certain easily accessible places in the cane field, to the west of the barracks, where the people lived.

5. Surveys of the pollution in the cane field showed a great reduction after the building of an adequate number of latrines and the carrying on of an educational campaign.

6. Examinations for infective hookworm larvae of soil samples from this area showed very little soil infestation, except along the heavily polluted strip of cane.

7. The records of the examination of the soil samples indicate that hard packed clay-loam soil on which there is no vegetation, even if moisture is present, does not offer conditions favorable for the development of hookworm larvae.

8. The localized character of soil infestation, especially in the cane field, showed that there was little migration of infective hookworm larvae from the places of development, although evidence is presented that they may be carried considerable distances by water.

9. The analysis of the habits of the people, in relation to the distribution of soil infestation, suggested that most of the heavy infestation of the people of this area was due to the habit which so many of them had of visiting certain places in the cane field for the purpose of defecation.

10. After the reduction of soil pollution in the cane field and the treatment of the people of the area, series of samples taken at intervals showed a rapid dying out of the infective hookworm larvae from this area, so that in about six weeks soil infestation was practically eliminated.

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INVESTIGATIONS ON THE CONTROL OF HOOKWORM DISEASE. VII.

An Epidemiologic Study of Hookworm Disease in a Cacao Estate.*

By WILLIAM W. CORT, Ph.D., and GEORGE C. PAYNE, M.D.

(Received for publication December 13, 1921.)

Introduction.

Since the production of cocoa is one of the chief industries of Trindad, British West Indies, it was of interest during our investigation there to study the conditions influencing the spread of hookworm disease on a cacao estate. The objects of this study were two-fold, viz.: (1) to locate the sources of human infestation and (2) to determine the reduction of soil infestation produced in localized centers of pollution in a cacao grove, after the treatment of the people who visited these spots.

Description of area.

For this study a part of an estate was chosen which was located about five miles from our laboratory. This area included two barracks and one detached dwelling, the yards surrounding the houses and that part of the cacao grove immediately back of the two barracks (see map). The cacao grove, which was thickly planted with full-grown trees and shaded by large immortel trees, was easily entered by two paths from the barracks. The detached house which was the home of the negro driver, his wife and five children, was in good repair, but the two barracks were quite dilapidated. The barracks were inhabited by East Indian laborers, of the same type as those on the sugar estate described in number six of this series (Cort and Payne, 1922*a*). In Barrack 1 lived five adult East Indians, one male and four females, and six children, while in Barrack 2 there lived an East Indian, his wife and four children. All these people had lived on this estate from four to seven years. The only animals on

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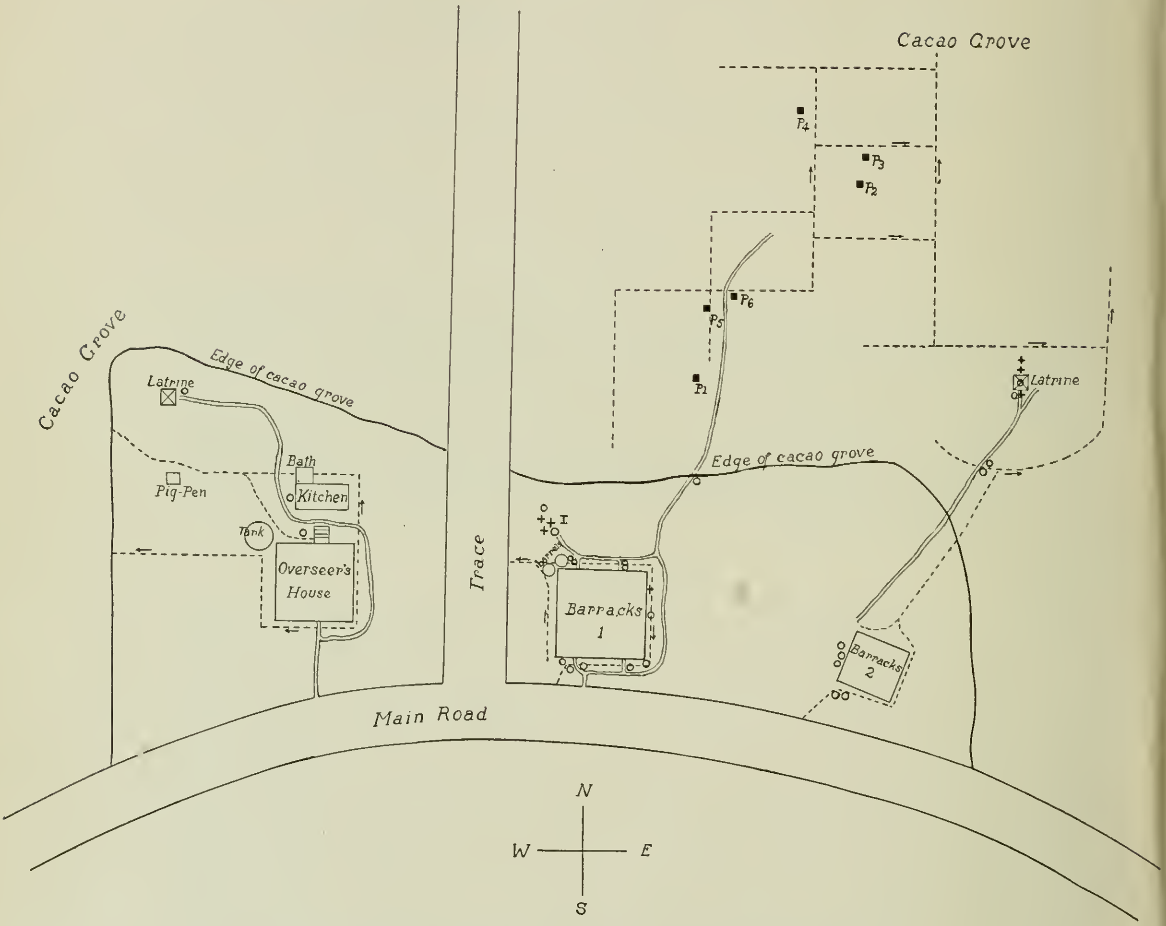
* This paper is the seventh of the series on the hookworm investigations of the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University, carried on with the cooperation of the International Health Board, Rockefeller Foundation.

MAP OF AREA OF CACAO ESTATE.



EXPLANATION OF MAP OF AREA ON CACAO ESTATE.

This map shows the relation between the barracks and the cacao grove in the area studied. The pollution centers in the cacao, from which soil samples were taken, are indicated at P_1–P_6. The paths are shown by two parallel lines and the drains by dotted lines. The position of the soil samples taken from near the houses and latrine, and the number of infective hookworm larvae present in these samples are indicated by the following signs:

No larvae = 0 1 — 10 = + 11 — 30 = I

the area were a pig, kept by the negro family, and a small number of chickens which ranged over the whole territory, around the houses and back into the cacao.

HUMAN INFESTATION.

There were only two light cases of hookworm disease in the negro family, the father and the eight-year-old son. Fifteen of the seventeen East Indians in the two barracks were infested with hookworms, the negatives being a girl of three and a boy of twelve. The infestations were heavy, except in the young children, and some showed distinct clinical symptoms. The people of this area were given three treatments by the method described in our previous paper (Cort and Payne, 1922*a*, p. 113), between July 23 and August 6. Only two of the positives, both East Indian women, escaped treatment, one by concealing herself and the other on account of an advanced pregnancy. Of the fifteen individuals treated, re-examinations were made of eleven, of which eight were still infested. From what is known of the effects of similar treatments elsewhere it can be argued that there was a great reduction of mass infestation following the treatments.

CONDITIONS OF SOIL POLLUTION.

No evidence of soil pollution was found around the house where the negro family lived. There was a well-constructed pit latrine near this house, which was kept in good condition, and used by all the members of the family.

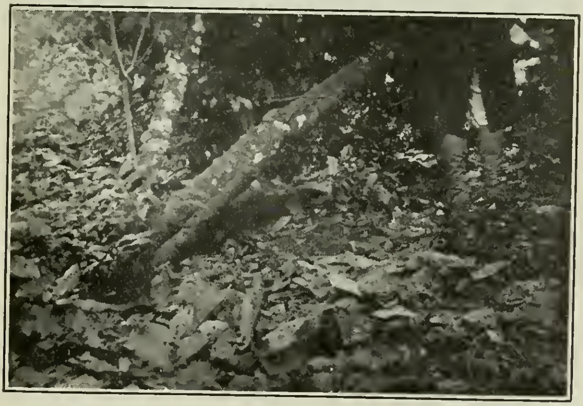


FIG. 1. A heavily polluted spot beside a leaning cacao tree (P_1).

There was an open-backed latrine back of Barrack 2, which was in filthy condition at the time of our early visits to this area (between June 6 and June 10). At this time it was evidently used by a few people, but later visits showed that its use had been entirely abandoned. Since this was the only latrine available to the people of the two barracks, it was evident that soil pollution was the rule.

Very little soil pollution was found near these two barracks. Some scattered stools were found at later visits around a banana tree, back of Barrack 1, and another pollution spot was found where the path from Barrack 2 entered the cacao. Inquiries brought out the statement that these places were visited only by children. It was also learned that the people did not wash themselves near the houses

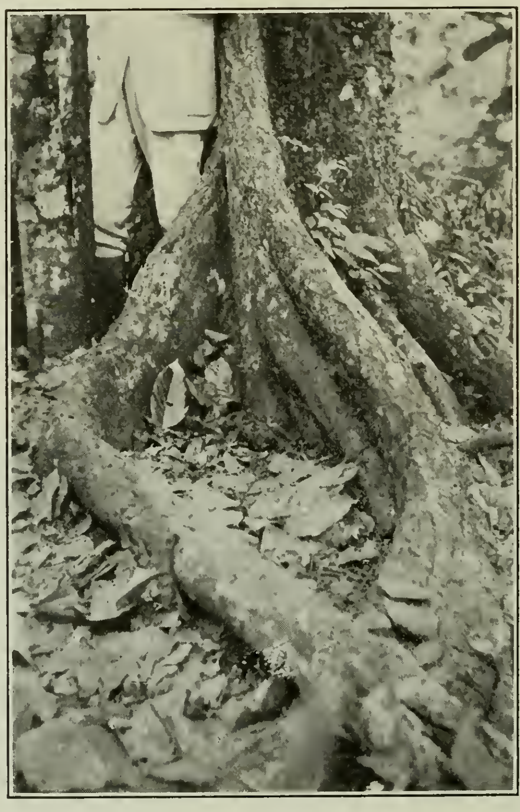


FIG. 2. A " natural latrine " entirely enclosed by the roots of an immortel tree (P_2).

after defecation, but carried bottles of water out with them into the cacao grove for this purpose.

In the cacao grove back of these two barracks there were definitely localized centers of pollution, which were visited day after day by the people of the barracks. At the time of the first visits to this place (June 4 to 20) there were found four of these pollution spots which constituted, as it were, natural latrines. The location of these pollution centers, which will be designated as P_1, P_2, P_3 and P_4, can be seen on the map. The spot designated as P_1 (Fig. 1) was at the side of a leaning cacao tree; P_2 (Fig. 2) was an area enclosed by the roots of a large immortel tree; P_3 (Fig. 3) was near P_2 at the side of the exposed roots of a cacao tree and P_4 (Fig. 4) was the area between the buttress roots of a second immortel tree. During the first series of surveys there was evidence of daily pollution at all these places. As in the polluted areas of the sugar cane field (Cort and Payne, 1922, p. 119), the stools disappeared quickly, chiefly from the action of the rain and the activities of insects, so that the places did not become foul smelling and their use seemed to the people much preferable to the use of the filthy latrine. That these " natural latrines " were in constant use is evidenced by the finding of pollution in them on visits made July 20 and 26 and later on September 3. At these later visits two new pollution centers were found around the roots of immortel trees

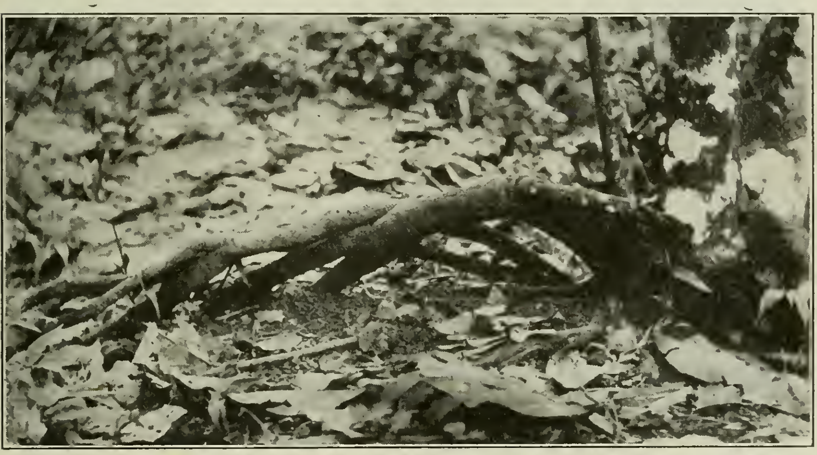


FIG. 3. A concentrated pollution center at the side of the exposed roots of a cacao tree (P_3).

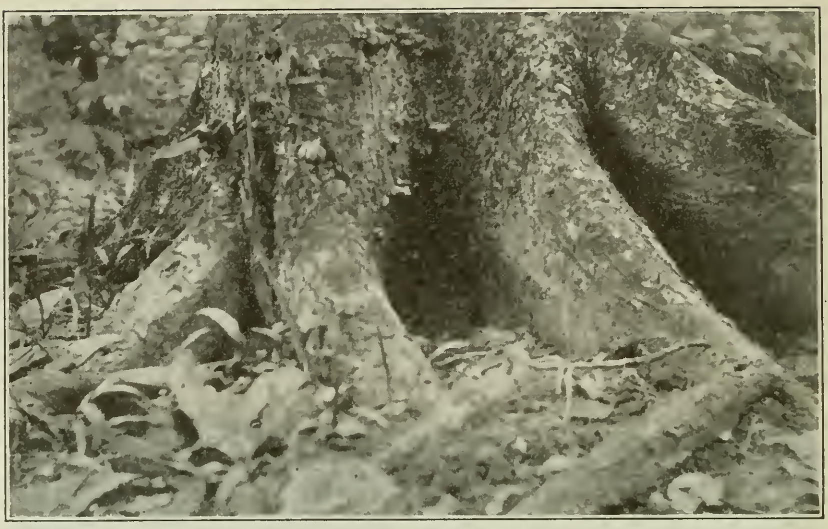


FIG. 4. A polluted spot between the buttress roots of an immortel tree (P_4).

(see P_5 and P_6 on the map). At P_5 the pollution was so great that there was formed between two of the roots a small area of liquified sludge, like that found in pit latrines, in which fly larvae were working. The pollution at these spots in the cacao was so definitely localized and they were so constantly visited that they may be considered as natural latrines, which served the people of these two barracks, and which seemed to them much cleaner and less objectionable than the ordinary pit latrines.

SOIL INFESTATION.

The study of soil infestation in this area was made by the use of the isolation apparatus (Cort, Ackert et al., 1921) in much the same way as in the investigation of the sugar estate previously described (Cort and Payne, 1922*a*). On June 13 and 17 a series of 12 soil samples were taken from immediately around the houses, from wet spots where the people washed themselves and their utensils, and from the drains. The results of the examination of all these samples for hookworm larvae were negative. About a month later seven more soil samples were taken from the same situations. Only one of these, a sample from a drain at Barrack 1, was positive, giving only one hookworm larva. The locations from which these nineteen soil samples were taken is shown on the map.

The other soil samples which were taken from this area, outside the cacao grove, are shown on the map. They were taken with relation to known soil pollution and show that centers of slight soil infestation had developed in the area polluted by the children, near the banana tree back of Barrack 1 and around the latrine.

Fifty soil samples were examined from the cacao grove back of Barracks 1 and 2, which were taken with relation to the pollution spots (P_1 to P_6). The surface of the ground in the cacao grove offered three different conditions, viz.: (1) bare clay soil, (2) clay covered by a layer of humus, and (3) that with the surface covered with a carpet of twigs and dead leaves (see Figs. 1 to 4). At first in taking the soil samples the surface leaves were brushed aside, but later it was found that the infective hookworm larvae were often found on the surface of the leaves, especially when they were wet with dew or rain (see Augustine, 1922*b*, p. 175). After this finding the soil samples were taken directly from the surface and consisted either of leaves or soil. Table 1 gives a complete list of samples taken from this environment.

TABLE 1.

Record of examination of soil samples from the cacao grove back of barracks 1 and 2. A. Leaning cacao tree (P_1).

No.	Date.	Location of sample.	Character of sample.	Larvae found.		
				Sheathed.	Un-sheathed.	Total.
28	June 13	At pollution center...	Surface humus.....	..	..	4
29	" 13	5 ft. W. of pollution center...........	Surface humus.....	..	..	0
38	" 15	At pollution center...	Leaves and humus .	12	16	28
40	" 15	At pollution center...	Surface clay and humus..........	..	..	0
63	" 17	At pollution center...	Leaves only.......	26	7	33
64	July 20	At pollution center...	Humus...........	96	30	124
65	" 20	1 yd. W. of pollution center............	Leaves and humus.	24	4	28
66	" 20	1 yd. N. of pollution center............	Leaves and humus.	26	8	34
67	" 20	Leaves around edge of cleared pollution center............	Leaves............	345	25	370
100	" 26	2 yds. W. of pollution center...........	Leaves and humus.	3	20	23
101	" 26	4 yds. W. of pollution center............	Leaves and humus.	..	..	0

B. Root enclosure at largest immortel tree (P_2).

No.	Date.	Location of sample.	Character of sample.	Larvae found.		
				Sheathed.	Unsheathed.	Total.
3	May 31	At pollution center within roots.......	Surface clay and humus..........	..	..	8
30	June 13	At pollution center within roots.......	Surface clay and humus..........	..	..	24
31	“ 13	2 ft. S. of pollution center............	Humus...........	..	..	0
37	“ 15	At pollution center...	Leaves............	..	..	1
42	“ 15	“ “ “ ...	Moist twigs.......	..	..	147
61	“ 17	“ “ “ ...	Leaves............	5	3	8
68	July 20	“ “ “ ...	Surface clay, humus and leaves.......	1	2	3
69	“ 20	In front of root just outside of pollution center............	Leaves and humus.	1	1	2
104	“ 26	At pollution center...	Leaves and soil....	..	1	1

C. At foot of cacao tree, seven feet north of P_2 (P_3).

No.	Date.	Location of sample.	Character of sample.	Larvae found.		
				Sheathed.	Unsheathed.	Total.
32	June 13	At pollution center...	Surface soil........	..	..	3
33	“ 13	At other side of tree, just out of pollution center............	Surface clay.......	..	..	0
62	“ 17	At pollution center...	Leaves............	16	47	63
70	July 20	“ “ “ ...	Leaves and humus.	19	14	33
71	“ 20	2 yds. E. of pollution center............	Leaves, humus and clay............	..	..	0
105	“ 26	At pollution center...	Leaves and humus.	1082	28	1110

While a large number of infective hookworm larvae were found the numbers were not in proportion to the concentration of the pollution. The finding of large numbers of unsheathed infective hookworm larvae in these samples need not be discussed here, since this point was covered in an earlier number of this series. (Cort, Augustine et al., 1922.)

D. At base of immortel tree, 25 feet northwest of P_2 (P_4).

No.	Date.	Location of sample.	Character of sample.	Larvae found.		
				Sheathed.	Un-sheathed.	Total.
34	June 13	At pollution center...	Surface clay	.	..	0
35	" 13	" " " ...	" "	..	..	1
43	" 15	" " " ...	Leaves and humus	..	..	0
72	July 20	" " " ...	" " " .	1	1	2
73	" 20	At base of small cacao, two yards east of pollution center...........	" " "	..	..	0
74	" 20	At edge of drain, 1 yard from pollution center...........	" " " .	..	..	0
75	" 20	At head of small drain, 1½ yards from pollution center.......... .	" " " .	2	1	3
76	" 20	3 yards down drain ..	" " " .	..	..	0
77	" 20	4 yards down drain ..	" " " .	..	..	0
78	" 20	6 yards down drain ..	" " " .	..	..	0
79	" 20	9 yards down drain ..	" " " .	..	..	0
80	" 20	2 yards south of No 73...............	" " " .	..	..	0
81	" 20	3 yards southwest of No. 73...........	" " " .	..	..	0
106	" 26	At pollution centers..	" " " .	5	15	20
107	" 26	Same as No. 73.....	" " "	..	4	4

The results of the examinations of the soil samples from the neighborhood of the leaning cacao tree (P_1) (Fig. 1) are given in Table 1, *A*. As was expected from the constant pollution at this place, there was found a high percentage of positives with numerous hookworm larvae. Some distribution of larvae out from the pollution center is indicated by the findings from samples 65, 66, 67 and 100. But that such soil infestation was not widespread is shown from samples 29 and 101. The slight spreading of the larvae from this pollution center could be easily accounted for by the scratching of chickens which frequently visited this place.

The results of the examinations of the samples from P_2 (Table 1, *B*) were very surprising, when it is considered that in an area about six feet long and two feet wide, enclosed by buttress roots

(Fig. 2), stools were apparently deposited daily by two or three people, at least one of whom was known to be heavily infested with hookworms. The soil within this area succeeded in absorbing the pollution without the formation of sludge. That such few larvae were isolated from the soil of this place indicates that for some reason or other the conditions here were not favorable for the development of hookworm larvae.

The findings from P_3 (Fig. 3) which are given in Table 1, *C*, indicate the development of intense soil infestation both on the leaves and the surface of the soil. The results of the examination of sample 71 show that there is very little migration under the conditions in the cacao, even from centers of intense soil infestation.

E. At base of immortel tree, 22 feet north of P_1 (P_5).

No.	Date.	Location of sample.	Character of sample.	Larvae found.		
				Sheathed.	Un-sheathed.	Total.
82	July 20	2 yds. in front of pollution center......	Leaves and humus.	..	..	0
83	" 20	At pollution spot....	" " " .	1	3	4
84	" 20	At edge of drain 1 yd. east of pollution spot.............	" " " .	..	..	0
85	" 20	At edge of drain 1 yd. north of pollution spot.............	" " " .	..	..	0
86	" 20	In drain 1½ yards east of pollution spot...	Clay..............	..	..	0
87	" 20	2 yds. west of pollution spot.........	Leaves and humus.	..	3	3
102	" 26	At pollution spot....	" " " .	2	3	5

F. At base of immortel tree, 30 feet north of P_1 (P_6).

No.	Date.	Location of sample.	Character of sample.	Larvae found.		
				Sheathed.	Un-sheathed.	Total.
103	July 26	At pollution center...	Leaves and humus.	135	106	241

The data from the examination of the soil samples taken from areas P_4 and P_5 (Table 1, *D* and *E*), indicate, either conditions unfavorable for the development of hookworm larvae, or pollution by negative or lightly infested individuals. The results from sample 103 (Table 1, *F*) show that a center of soil infestation had developed at P_6.

The results of the examination of soil samples in the cacao (Table 1) show that centers of soil infestation had developed at certain polluted spots. These results are surprisingly irregular, when the constant pollution of these places, and the heavy infestation with hookworms of the people visiting them are considered. It is impossible to explain these irregularities from the data at hand. Three possible factors suggest themselves which might be responsible, viz.: (1) the presence of leaves on the soil which complicated the taking of the soil samples; (2) possible variations in the degree of infestation of people visiting the different pollution centers; and (3) the development at certain of these places of conditions unfavorable to the development of hookworm larvae. Certain activities of the infective hookworm larvae in the cacao environment have been studied by Augustine (1922, p. 175). His findings that the larvae do not migrate any distance under conditions existing in the cacao grove and that they have a tendency to get onto the upper surfaces of the leaves were verified by our field studies.

Sources of human infestation.

The data given above indicate that the only really important sources of infestation with hookworms in this area are the centers of soil infestation developed at the highly polluted spots in the cacao. The area around the banana tree, back of Barrack 1, might have been responsible for some infestation, especially in children, and the slight soil infestation in front of the latrine might have given a few worms to those visiting it. However, the soil infestation at the polluted centers back of the barracks, the constant visiting of these places by the East Indians, and the fact that this part of the cacao was known as a ground itch area, suggests that from here most of the hookworm infestation found in Barracks 1 and 2 was derived. Further evidence for this view is given by the finding that all the negro family were negative, except the driver and his eight-year-old son. It was found by inquiry that the negro boy had acquired ground itch from playing in the cacao back of the East Indian barracks, and that the driver, who wore heavy boots, had had ground itch on his hands from working in this area. Therefore, here again as in the sugar estate (Cort and Payne, 1922, p. 142) it was found that the soil pollution habits of the people were chiefly responsible for their hookworm infestation, especially when they were accustomed to visit the same place day after day.

Reduction of soil infestation following treatment.

We decided to try out in this area the reduction of soil infestation produced by three routine treatments, when there had been no reduction of soil pollution. The records of treatments given above show that two people were untreated and that of the eleven examined after treatment eight were still positive. Therefore, while a considerable reduction of mass infestation must have resulted from the treatments, there was still left an appreciable infestation of the majority of those treated. Table 2 shows by a comparison of the results of the examination of soil samples, taken July 20 and 26, from the centers of pollution in the cacao, with a series from the same places taken September 3, that this reduction of mass infestation was quickly reflected in the reduction of soil infestation. The samples of September 3 were taken just after a rain and consisted of the scrapings from the entire surface of the polluted spots. Since the larvae have been shown to come to the surface after it is moistened (Augustine,

TABLE 2.

Comparison of samples from polluted spots in the cacao grove (P_1–P_6), *before and after treatment.*

Sample No.	Location.	Ex. July 20 and July 26.			Ex. Sept. 3d.		
		Sheathed.	Un-sheathed.	Total.	Sheathed.	Un-sheathed.	Total.
64	P1. Cleared place at center...........	96	30	126	2	19	21
67	P1. Leaves around cleared place.....	345	25	370	1	9	10
105	P3................	1,082	28	1,110	26	23	49
106	P4................	5	15	20	..	..	0
102	P5................	2	3	5	..	..	0
103	P6................	135	106	241	1	8	9
Total....		1,665	207	1,872	30	59	89

1922*b*) these samples were taken under the most favorable conditions possible for locating infective hookworm larvae. This fact makes the great reduction in numbers of larvae shown by Table 2, all the more significant. These findings show that in areas of soil infestation in the cacao cultivation the length of life of infective hookworm larvae is short.

SUMMARY.

1. Examination of the people living in three houses on a cacao estate showed a heavy infestation with hookworms.

2. Soil pollution in this area was almost entirely restricted to definite spots—" natural latrines " in the cacao grove near the barracks.

3. The examination of soil samples showed very little soil infestation anywhere in the area, except at the " natural latrines " in the cacao, and the conclusion can be drawn that almost all the human infestation must have come from the habit of polluting the soil of the cacao.

4. Even in the "natural latrines" the findings were somewhat irregular, indicating the conditions were not always favorable for the development of hookworm larvae, and that they did not migrate from the place of development.

5. Examinations of the soil of the intensely polluted spots in the cacao, six weeks after three routine treatments had been given to the people, showed a very marked reduction of soil infestation, indicating that in this situation the life of the infective hookworm larvae is short.

For bibliography see No. VI of this series.

INVESTIGATIONS ON THE CONTROL OF HOOKWORM DISEASE. VIII.

Experiments on the Migration of Hookworm Larvae in Soils.*

By DONALD L. AUGUSTINE, B.S.

(Received for publication December 13, 1921.)

Historical account.

Until Baermann (1917) devised an apparatus with which hookworm larvae could be successfully isolated from the soil, little or nothing was known of their activities under natural conditions. Throughout the literature, one is impressed with the idea that the infective hookworm larvae may actively migrate for considerable distances, and may thus give rise to infestation far from the place where the eggs were originally deposited.

Claude A. Smith (1903, p. 710) recorded that the larvae dart through a sandy soil at a lively rate for a few minutes to half an hour or more, and then straighten out and lie perfectly still for a corresponding length of time. In the report of the Porto Rico Anemia Commission (1904, p. 121) the statement was made that rain causes a distribution of larvae, and that this, together with their natural motility, may spread nests of soil infestation over a considerable area. Nicoll (1917, p. 163) in his discussion of the hookworm larvae, under natural conditions, made the following statement:

" The larvae now measure about 0.6 mm. in length and are very actively motile. Unless they have been washed away previously from the faeces, they now proceed to migrate to a more suitable locality, namely: the nearest water pool or patch of moist ground. Provided even a trace of moisture be present, they are capable of traversing considerable distances and may thus give rise to infection far from the place, where the faeces were originally deposited. How far and how rapidly they may wander in this fashion is not definitely known, but judging from laboratory experiences their rate is probably not less than five feet per hour, so that in the course of 24 hours, they may have wandered 40 yards. This means that an area of 5,000 square yards might become infected within 24 hours after the larvae started to migrate, i.e., within a week after the faeces were deposited."

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* This paper is the eighth of the series on the hookworm investigations of the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University, carried on with the cooperation of the International Health Board, Rockefeller Foundation.

Chandler (1918, p. 259) pointed out that continual traveling in a straight line would probably never occur, but that a single stool containing hookworm eggs could easily infest at least several square yards. Similar statements on the spread of hookworm larvae were also made by Ozzard (1909, p. 779).

The first intimation that the infective hookworm larvae might be limited in their distribution throughout the soil was made by Price (1910, p. 205). He stated that in his opinion sub-soil pollution was not an important factor in hookworm disease, because it appears inconceivable that hookworm larvae will pass for any considerable distance through a sand and gravel sub-soil.

Based on the work of these authors, the opinion has become current that the infective hookworm larvae move out from centers of infestation and spread themselves over wide areas. This conception is founded largely upon the study of the behavior of the encapsuled larvae when mounted in water, or water and soil, on glass slides or in watch glasses, and examined microscopically. To what extent the migrations of the larvae occur from definite infested centers in soils of various types and under varying conditions, has not yet been demonstrated. The experiments which will be described in this paper were carried out in Trinidad, British West Indies, from May to September, 1921, in order to gain information on this very subject.

I wish here to express my appreciation to Dr. W. W. Cort, under whose direction the investigations embodied in this and the two following papers were carried on, for his constant interest and helpful suggestions. Thanks are also due Dr. James E. Ackert, of the Kansas State Agricultural College, Dr. Florence King Payne and Dr. George C. Payne, Trinidad, for assistance in securing material and for suggestions during the course of the work.

Material and methods.

The larvae used in these experiments were obtained from cultures 5 to 8 days old. A detailed account of the method used in the preparation of the cultures is given in number two of this series (Cort, et al., 1922, p. 6). The larvae were carefully counted under the microscope, the 16 mm. objective being used, when the hookworm larvae were mixed with other nematodes, and a lower power, when they were in pure culture. As *Ancylostoma duodenale* is very rare in Trinidad it may be assumed that the larvae obtained were those of *Necator americanus.*

Two sizes of pans were used in the experiments on migration, viz.: (1) round tin dripping pans, 13 inches in diameter and 4 inches high, and (2) pudding pans 6 inches in diameter and 2 inches high.

The soils in which these larvae were studied were selected from the district in which these experiments were carried on, the Naparima District, which is in the south central part of Trinidad. They were chosen from areas free from soil pollution, and tested in the laboratory with the isolating apparatus for the presence of hookworm larvae and free-living nematodes. None of the samples were found to contain hookworm larvae, and where free-living nematodes were numerous, the soil was heated to 150° F. This temperature was found to be sufficient to kill all living organisms present without altering the composition of the soil itself.

The migrations of the hookworm larvae were studied in five distinct types of soils. Type 1 was a black, rather heavy, clay loam which contained no small stones. Type 2 was identical with type 1, except that it contained a greater amount of humus and was lighter. These two soils are classified by Williams (1921, pp. 106–109) under the Maparina Marls or the Black Soils of the Maparimas. Type 3 was also a heavy clay loam distinguished by its red color. It differed from types 1 and 2, chiefly in its chemical rather than mechanical composition being deficient in lime and having a high percentage of iron. Type 4 was a fine sand and clay mixture, light when dry or slightly moist, but heavy and compact when wet. Type 5 was a loose reddish sand, containing only a small amount of clay.

Experiments on active migration.

Table 1 gives the data from a series of twenty experiments to test the active migration of infective hookworm larvae under a variety of conditions. In all the experiments, summarized in this table, the soils were placed to the depth of 1 cm. in the 13-inch dripping pans. The center was then determined, marked by a cord circle 1 inch in diameter, and the larvae placed within this circle. This area was termed Zone 1, or the center of infestation. When I wished to close an experiment the soil was further divided into Zones 2, 3 and 4. This was accomplished with an apparatus similar to a doughnut or cooky cutter. It consisted of three stationary tin bands of sufficient circumference to make each of these zones two inches wide. The soils of the three outer zones were transferred separately to units of the large isolation apparatus, without withdrawing the "cutter." The danger of mixing the soil of one zone with the next was thereby reduced to a minimum. Two units of the isolation apparatus were used for Zone 4, to keep the amount of soil in each unit approximately equal. The cutter was then removed, and the soil from the infestation center transferred to a unit of the small isolation apparatus.

In describing the moisture content of the soils the term "moist" indicates a soil which had a slightly sensible wetness, while the term "saturated" denotes a soil which had been thoroughly soaked and its surface fairly covered with water.

It will be seen from an examination of Table 1, that the larvae did not manifest the migratory habit which has been attributed to them, since in no instance were they found as far as four inches from the center in which they were placed. In only 4 experiments (Table 1, nos. 5, 7, 17 and 19) were larvae recovered from the third zone, and in each of these cases less than 2 per cent. of the total number of larvae recovered had reached this zone. It is also apparent that neither the type of soil nor the presence of vegetation enter as factors in the extent of active migration of hookworm larvae. A thick grassy sod was not available at the time of these experiments, and in order to study the larvae under this environment the soil—clay loam—was first heavily sown with a mixture of oats and wheat. The larvae were not added to the infestation centers until the grass had formed a heavy root system, and had reached a height of 3 to 4 inches. No perceptible difference was observed in the extent of migration of the hookworm larvae, when exposed to direct sunlight under temperatures varying from 93° F. to 101° F. (Experiment 20, Table 1). The migration pan was set out in direct sunlight during the day and returned to the laboratory at night. At no time was the soil allowed to become dry. As soon as a dry crust appeared on the surface it was evenly sprinkled with water, thus keeping it as uniformly "moist" as possible. While the sunlight had no apparent influence on the extent of spread of the larvae away from the infestation center, it had an indirect effect on the activities of the larvae within that area which is discussed in number eight of this series.

TABLE 1.

Experiments on the active migration of hookworm larvae. All these tests were made in the thirteen-inch pans.

Experiment number.	No. larvae in center of infestations.	Type of soil.	Conditions.	Time larvae remained in soil.	No. of H.W. larvae recovered from			
					Zone 1.	Zone 2.	Zone 3.	Zone 4.
1	800	Clay loam	Moist, shade	15 hours	409	15	0	0
2	1,000	Clay "	" "	16 "	583	274	0	0
3	1,800	Loose loam	" "	40 "	1,395	128	0	0
4	1,000	Loose "	" "	15 "	460	406	0	0
5	1,000	Clay loam	" "	7 days	236	84	1	0
6	1,000	Clay "	" "	7 "	478	39	0	0
7	512	Clay "	" "	11 "	89	145	3	0
8	1,100	Clay "	" "	21 "	13	5	0	0
9	650	Clay "	" "	42 "	1	0	0	0
10	525	Red clay loam	" "	15 hours	363	69	0	0
11	500	" " "	" "	22 days	6	3	0	0
12	277	Clay loam sod	" "	7 "	60	10	0	0
13	392	" " "	" "	21 "	2	3	0	0
14	361	" " "	" "	30 "	1	1	0	0
15	426	" " "	" "	37 "	1	0	0	0
16	498	Sand	" "	10 "	51	40	0	0
17	451	Sand	" "	22 "	77	16	1	0
18	498	Sand	" "	29 "	51	40	0	0
19	1,300	Sand and clay	" "	8 "	199	174	5	0
20	500	Clay loam	Moist, direct sun	35 "	5	0	0	0

Early in these experiments it was noted that there occurred a remarkable reduction from the original number of larvae placed in the infestation centers, even after a week's time. It will be noted, from Table 1, that this reduction increased with the number of days the larvae remained in the soil. Here, as well as in other experiments made, a large percentage of the larvae recovered had lost their sheaths.

This experimental evidence on the failure of the hookworm larvae to migrate in the soil is supported by similar findings of Cort and Payne (1922*a*, p. 137), in their study on the sources of hookworm infestation in a cane field. Instead of finding a uniform distribution of the larvae over the total area studied, they were found to be definitely localized at the places of soil pollution. Often where two soil samples were taken close together one would contain a large number of larvae, while the other would contain only a few or be even negative. As a specific example, approximately 1,000 hookworm larvae were isolated from a pint of soil taken from a pollution spot 15 yards into the cane. Pint samples taken from the areas within a radius of 1½ feet to 2 yards from this yielded from 0 to 53 hookworm larvae per sample. The environment here was seemingly in every way favorable for migration, yet during the entire time of the investigation, the larvae were always found at more or less definitely localized spots, at the places of soil pollution. That there occurs little or no migration of the hookworm larvae even from centers of very heavy soil infestation has also been shown by Cort and Payne (1922*b*, p. 159) under conditions existing in a cacao grove. The evidence from these field studies and the data from the laboratory experiments given above show that it will be necessary to revise our present conception of the extent of the migrations of infective hookworm larvae, and make it seem very probable that it is only by mechanical means that hookworm larvae can spread from the place of their development.

WHEN PLACES OF SOIL INFESTATION BECOME DRY, DO HOOKWORM LARVAE MIGRATE TO MORE FAVORABLE SITUATIONS?

To test this point, a large number of hookworm larvae were placed on a small spot in a 13-inch dripping pan, containing moist sand 1 cm. in depth. A second area, to which no larvae were added, was located 8 inches away from the first. The whole surface was then evenly sprinkled with water. Water was afterwards added daily to the second area which alone was kept constantly moistened. Ten days later all the sand appeared to be thoroughly dry except that in and around the watered area. This was transferred to a unit of the small isolating apparatus, and upon examination, proved to be negative for hookworm larvae. A small amount of soil taken directly from the original infestation spot was placed on a glass slide, a few drops of water were added, and upon microscopial examination three shrivelled, completely disorganized hookworm larvae were found. The remaining soil of this area was then examined, but yielded no larvae. This experiment indicates that infective hookworm larvae cannot migrate from a drying area to a moist area nearby, but, of course, needs confirmation by a larger series of experiments.

Experiments on passive migration.

In two experiments made in the same manner as those summarized in Table 1, except that water was added until the surface of the soils was covered, a fairly uniform distribution of the larvae was found in all zones. In the first, with clay loam soil, where 500 hookworm larvae were added to the infestation center, 72 larvae were recovered from that area, 70 from the second, 17 from the third and 3 from the fourth, eight days after the experiment was started. In the second, with a sand and clay soil, 1,300 larvae were placed in the center of infestation. Eight days later 83 larvae were recovered from Zone 1, 271 from Zone 2, 245 from Zone 3 and 258 from Zone 4.

As these two above experiments differed from those of Table 1 only in the amount of water added, it seemed that in these cases the larvae might have been mechanically carried out of the infestation centers by the surface water. In fact, in the examination of preparations in the counting of larvae, they were often found on the surface film.

To determine the numbers of larvae that might be carried from a given center by this means, the following experiments (see Table 2) were made. Two hundred and twenty-two active hookworm larvae were placed in the center of a 6-inch pudding pan, containing one inch of clay loam soil. The larvae remained undisturbed for several minutes when water was added, until the soil was well covered. The standing water was immediately drawn off with a pipette from all parts of the surface, except that in which the larvae had been placed. This was centrifugated and upon examination of the residue 52 hookworm larvae were found. This showed that 23.4 per cent. of the total number of larvae, placed in the infestation center, had been

TABLE 2.

Showing number of hookworm larvae carried in surface film.

Exp. No.	No. larvae used.	Position of larvae in soil.	No. larvae recovered.	Per cent. recovery.
1	222	No larvae visible..................	52	23.4
2	234	No larvae visible..................	61	26.1
3	100	No larvae visible..................	28	28.0
4	268	Larvae protruding from surface.......	117	43.6
5	127	Larvae protruding from surface.......	38	29.9
6	156	Larvae protruding from surface.......	57	36.5

carried out by the water. Experiments 2 and 3 of this table were made under the same conditions as the first, and their results further illustrate the rôle water plays in the dissemination of hookworm larvae.

In the first three experiments of Table 2 no larvae were visible on the surface of the soil, at the time the water was added. In experiments 4, 5 and 6 of the same table, the larvae had been placed in the soil 15 hours before the water was added. During that time they had established themselves on the surface of the soil, and were found protruding from the prominent soil particles within the infested area. Water was added as before, until the soil was well covered, and upon examination of the residue from the free water slightly higher percentages of the larvae were recovered than in the first three experiments (see Table 2). Apparently when the larvae are well established on the surface of the soil, they are more easily transferred to the surface film.

That hookworm larvae are carried away from centers of soil infestation by the washing of rains, under natural conditions, is indicated by the findings of Cort and Payne (1922, p. 141). Soil samples examined from the drains in the heavily infested area of the cane field studied, showed that the larvae were fairly evenly distributed for a distance of 50 yards. Soil pollution was found only in the higher parts of the drains and their environs. The authors consider that this distribution in the drains is due to the larvae being washed down from the upper ends of the drains, as well as from the areas drained.

To determine the number of hookworm larvae carried on the feet of man, after passage through areas of heavy soil infestation, the soil was scraped from the shoes of three persons, after they had completed a pollution survey in a cane field (see Cort and Payne, 1922*a*, p. 115). Soil obtained in this way was examined for hookworm larvae by the routine method, on six different occasions. The findings varied from 2 to 344 hookworm larvae per person. No appreciable difference was noted in the number of larvae obtained, whether rain had fallen twenty-four hours previous to the survey or not. This is explained by the fact that although the amount of rainfall varied, the soil in the cane was always moist enough to cling to the shoes in considerable amounts. The finding of 344 hookworm larvae in the soil adhering to the shoes of one person is significant. This person had undoubtedly stepped in a "nest" of larvae which was immediately transferred to his shoe. Had he been barefoot and the mud remained on the skin, the resulting infestation would probably have been heavy from this one exposure. Again, had the soil been washed off or dropped on a frequented path, there would have been danger to others.

Domestic animals passing through infested areas likewise enter as factors in the dissemination of hookworm larvae. No actual experiments were made to determine this point, but, from observations made, both in cacao groves and cane fields, it seemed evident that a considerable amount of infested soil may be scattered about in this fashion.

The results of my experiments, as well as field observations, have shown that the hookworm larvae are not migratory during their free life in the soil of Trinidad, but that they can be spread to some extent mechanically. Further investigations are needed to test out, under a variety of conditions, the points brought out in this study.

SUMMARY.

1. Experiments carried on in Trinidad, British West Indies, from May to September, 1922, showed that infective hookworm larvae placed on moist soils do not migrate in periods from 15 hours to 42 days.

2. Experiments show that hookworm larvae may be carried out from centers of soil infestation by surface water, and that they can establish themselves in the new locations when the water recedes.

3. Hookworm larvae were not found to migrate to favorable situations when their original environments become unfavorable.

4. Soil scraped from the shoes of men passing through infested areas was found to contain hookworm larvae. It is possible that paths leading to such areas, as well as the immediate surroundings of dwellings, become centers of infestation by the dropping of such soil.

5. During the time the larvae remained in the soil there occurred a reduction in their numbers, which increased with the number of days the experiment lasted.

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INVESTIGATIONS ON THE CONTROL OF HOOKWORM DISEASE. IX.

On the Position of the Infective Hookworm Larvae in the Soil.*

By DONALD L. AUGUSTINE, B.S.

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While it is known that the soil is the natural habitat of the infective hookworm larvae, only a few observations have been made as to their activities and position in it. Baermann (1917, p. 622), in his studies of the conditions of development and existence of hookworm larvae in the soil, found them to be rather evenly distributed throughout the soil to a depth of 24 cm. He did not find them directly on the surface but close to it. In other experiments, where larvae were studied in soil overgrown with vegetation, he found hookworm larvae constantly in the soil, but never on the plants themselves. No information is given on the exact conditions existing at the time his observations were made. The data from a series of experiments performed by Dr. James E. Ackert, to study the conditions under which hookworm eggs develop and hatch, show that by far the greater number of the hookworm larvae which developed were in the upper half inch of the soil. The complete discussion of these experiments will appear in a later number of this series. Looss (1905, English translation given in Looss, 1911, pp. 421–422) observed mature Ankylostoma larvae on the surface of charcoal cultures when under a high temperature and in a moist atmosphere. He found formations resembling short fungal hyphae, welded together, which upon examination proved to be hookworm larvae. He further observed that the larvae were not present on the surface when it became drier, but upon addition of water would reappear in 20 to 30 seconds. Smith (1905, p. 192) records the same position of the hookworm larvae on the surface of feces, when exposed to the air for a few days under favorable conditions of moisture. The

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* This paper is the ninth of the series on the hookworm investigations of the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University, carried on with the cooperation of the International Health Board, Rockefeller Foundation.

Georgia State Board of Health (1910) makes the statement that during a rain, or when dew drops collect, hookworm larvae find their way into the water where they remain, if the water does not dry up, until they have an opportunity to penetrate the human body. And when the dew drops dry or the pool of water evaporates the larvae burrow into the moist earth, there remaining until a fresh downpour of rain brings them to the surface again. Dock and Bass (1910) in their book on hookworm disease say that the larvae may crawl up the leaves, stalks or fruit of vegetables. No statement is made of exact observations of larvae doing this. Schultz (1912) found that in cultures from which blades of grass, small sticks, or other objects project, upon which a film of water condenses, hookworm larvae leave the cultures, follow the water film and ascend to the most advantageous point for contact with an eventual host.

Early in the experiments performed in Trinidad, British West Indies, on the migration of hookworm larvae in the moist loam soils (Augustine, 1922) I found them on the surface extending from the larger and more prominent soil particles. The greater number appeared singly like the hairs on young plants, while others were massed together in flame-shaped formations. Upon close examination with a hand lens the larvae seemed to be in constant undulating movement which greatly increased when the slightest stimulus was applied. A portion of the soil upon which several larvae were extended was placed on a glass slide and treated with a few drops of water. Upon microscopical examination both sheathed and unsheathed larvae were found which left the soil and moved with great rapidity to the margins of the preparation. The bit of soil was then broken and spread over the slide. In it several cast-off hookworm sheaths were found. The larvae on the surface of the soil readily transferred themselves to most objects that touched them, but not to insects, such as ants, as well as small Arachnids which passed through and about the infested centers.

Larvae were found on the surface of the soil in all my experiments, as long as their numbers were sufficiently large for observation and the soil was kept moist. In an experiment to test the effect of direct sunlight on the migrations of hookworm larvae (Augustine, 1922, p. 165) they were found extended from the soil particles as long as the soil remained moist, even at a temperature of 101° F. and in direct sunlight. As the surface of the soil became drier they left their more prominent positions and were found in the deeper cracks and crevices, disappearing altogether if the drying continued. If water was then added the hookworm larvae reappeared in a few seconds in their previous positions. This process was repeated several times a day with the same result, until their numbers became so reduced that the reactions could not be observed. It seems clear from this exeriment that sunlight in itself has no effect upon the movements of the larvae, but influences them indirectly by raising the temperature and increasing the rate of evaporation.

In two experiments on the migration of hookworm larvae (Augustine, 1922, p. 168), where water covered the soil, hookworm larvae appeared extended from short sticks, pebbles and larger soil particles which protruded above the water. They were usually motionless, but the application of a slight stimulus, such as blowing over the surface or jarring the pan, would start them to intense activity, especially where they had formed themselves into petal-like clumps. Toothpicks were placed upright in the soil in the infestation centers. A few hours later the larvae were found one third of the distance up the toothpick, but only as far as the moisture had ascended.

During the study of the active migrations of hookworm larvae in grass-covered soil (Augustine, 1922 p. 165) observations were made to discover whether the larvae would climb upon the blades of grass and collect in drops of water, as has been suggested might happen in morning dew. With a hand lens larvae were found at the bases of the stems and on the soil within the centers of infestation. They were also found along the outer dead sheaths of the grass which were moist. but never on the green stalks and blades. The green stalks and blades were cut from and surrounding the infestation centers and washed well in water. This water was then centrifugated and the residue examined with the microscope. No hookworm larvae were found. This test was repeated several times with constantly negative results. The probable reason for the larvae not creeping up the green stalks and into drops of water collected in the axiles of the leaves is that there was no continuous film of moisture present as on the dead stalks and sticks. Their movements seem to be definitely limited by the extent of the moisture film.

In connection with a study carried on by Cort and Payne (1922, p. 149) to determine the sources of infestation with hookworm on a cacao estate an attempt was made to duplicate in the laboratory the conditions existing in this environment, to determine the activities of the infective hookworm larvae under such conditions. In a particular grove of this estate the subsoil was a fine, compact, yellow clay. The surface soil contained an abundance of humus and was of a loose texture and dark. Scattered over much of the surface was a layer of decaying twigs and cacao leaves which during the rainy season were constantly moistened by intermittent showers. Here and there the surface of the yellow clay was exposed, and in other places the humus was free from the leaves and twigs. Soil pollution and infestation was found on all three types of surfaces. The soils, twigs and leaves taken for the laboratory experiment were selected from places widely separated from polluted areas. The yellow subsoil was first placed to a depth of 1¼ inches in a circular pan, 3 feet in diameter. Two thirds of its surface was then covered with the humus, and half of the humus area was carpeted with the leaves and twigs. At the center of each of these surfaces were placed approximately 1000 hookworm larvae, their positions being marked by circles of cord. On the following day a careful examination was made with a hand lens. Numerous larvae were found extended from the soil particles within the infestation centers on the yellow clay and humus soils. The larvae which had been placed upon the leaves were not confined within the center, but were found distributed within a radius of eight inches. They were found on the center margins and prominent veins of the leaves and on decaying bark of twigs, especially where the bark was broken and distinct edges were formed. It is probable that the greater extent of distribution of the larvae on the leaves was not due entirely to their active migration, but was aided by the washing of the water during the process of moistening; for while the water which was added was immediately absorbed by the two soils, it ran off the leaves, from one to another. Larvae were found within the infestation centers of the two soil surfaces at all times until their numbers became greatly depleted. The leaves lost their moisture quickly, and when dry the larvae disappeared to the deeper moisture places, returning to their former position at the next watering.

The position of the hookworm larvae, as found in these and other experiments, is significant since it makes easy the transfer of the larvae from the soil and other infested objects to the human foot.

SUMMARY.

1. Infective hookworm larvae under optimum conditions of moisture and temperature were found to remain on and within the upper surface of the soil.

2. They creep up pieces of wood, decaying vegetation and other objects only as far as the film of moisture extends.

3. Hookworm larvae were not found within the drops of water collected in the axile of the leaves of green plants nor upon the leaves themselves.

4. At centers of soil infestation, where the surface is covered with leaves or twigs, the infective hookworm larvae were found extended from the leaves or twigs when moist, but in the soil beneath when dry.

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INVESTIGATIONS ON THE CONTROL OF HOOKWORM DISEASE. X.

EXPERIMENTS ON THE LENGTH OF LIFE OF INFECTIVE HOOKWORM LARVAE IN SOILS.*

BY DONALD L. AUGUSTINE, B.S.

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HISTORICAL ACCOUNT.

The conception of the length of life of the infective hookworm larvae in the soil which is widely accepted can be illustrated by a quotation from Nicoll (1917, p. 163): "These larvae (hookworm), when they reach this final stage have ceased to feed, and they can remain alive for months and even years under suitable conditions, i.e., where there is a sufficiency of moisture and not too great heat. In the laboratory they have been kept alive for over 18 months in plain water, at a temperature of about 60° F. It can hardly be doubted that they will live fully as long under natural conditions, unless it be that they are attacked and devoured by other animals, such as aquatic insects." The evidence which points to an extended period of life for the mature hookworm larvae comes chiefly from the work of the first seven investigators listed in Table 1. These observations (see Table 1) were made upon sheathed larvae, kept in moist feces or water under laboratory conditions in the temperate zone. Further, the inference has often been drawn, as in the above quotation, that if the larvae can remain alive for such periods of time under unnatural surroundings, their span of life would be as great or even greater, when in their natural environment.

The work of the last three investigators summarized in Table 1 needs further discussion, since their observations were made under natural conditions. Leichtenstern (1887, p. 669) found the larvae of *Ancylostoma duodenale* alive in the fecal mass from a brick-yard

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* This paper is the tenth of the series on the hookworm investigations by the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University, carried on with the cooperation of the International Health Board, Rockefeller Foundation.

latrine three months after the laborers had left the field. Stiles and Gardner (1910) found two motionless, but well-preserved sheathed hookworm larvae in sand 151 days after the sample had been taken from under a surface privy. In experiments by these investigators, where fecal material containing hookworm eggs was covered with sterilized sand, examinations made four and five months later disclosed no hookworm larvae. These experiments were carried on at unheated room temperature in the early fall at the United States Marine Hospital Station, Wilmington, North Carolina. Baermann (1917, p. 622) in his studies on the conditions of development and existence of hookworm larvae in the tropical climate of Sumatra found that the length of life varied under different conditions of moisture and shade. In one box, where a stool containing hookworm eggs was covered with earth by a sexton beetle and exposed to sun and rain without additional moistening, some slight development took place, but the larvae were

TABLE 1.

Showing results of previous authors' observations on length of life of hookworm larvae.

Authors.	Medium.	Environmental conditions.	Duration of life.
Leichtenstern, 1887	Water Moist feces	Laboratory temperature in Germany	7 months
Galli-Valerio, 1905	Moist feces	Laboratory temperature in Switzerland	13 months
Boycott, 1905.	Moist feces	Laboratory temperature in London, England	More than 12 months
Loebker and Bruns, 1906.	Water	By laboratory window, Germany	12 months
Oliver, 1910	Water	Laboratory temperature, England	15 months
Perroncito, 1913 . .	Water	Laboratory temperature, Italy	8–10 months
Nicoll, 1917	Water	60° F.	18 months
Leichtenstern, 1887	Fecal material	Abandoned latrine in brickyard, Germany.	3 months
Stiles and Gardner, 1910	Sand	Unheated laboratory during early fall, Wilmington, N. C.	A few alive up to 4 months
Baermann, 1917. . .	Soil covered with sod	In the open shade, Sumatra	6 months

found to have died after three weeks. On shaded, sprinkled soil the larvae developed in large numbers, lived for 3 months and then died. In boxes planted with grass extensive development of larvae took place in both sun and shade. He found numerous larvae in the unshaded soil up to three months, and a few up to six months in the shaded. The results of these experiments on the length of life of hookworm larvae in the soil, with conditions as nearly natural as possible, gave a shorter period of life than the others in which the conditions were more unnatural, and suggest that soil does not remain infective as long as has been generally believed.

Leuckart (1868, p. 436), many years ago, pointed out that the mature larvae of *Ancylostoma caninum* (*Dochmius trignocephalus*) live at the expense of reserve food stored in the cells of the intestine during development, and as time passes these cells gradually loose their granules, until the worms become transparent. Looss (1911, p. 419) concluded that if the granules deposited in the body are reserve food stores, which are gradually reabsorbed during the later life of the larvae, a limit is set to the length of life, and death will come when this supply is exhausted. He believed that the length of life would depend on whether the supply of food was consumed slowly or rapidly, and since temperature influences to a high degree the motility of the mature larvae, they would remain alive longer at a low than a high temperature. If we accept this view we should expect the life of the larvae to be the shortest under those conditions of temperature which are most favorable to intense activity, as in the tropics. The results of my experiments carried on in Trinidad, British West Indies, and the field studies of Cort and Payne (1922*a* and *b*) support this view.

MATERIAL AND METHODS.

The hookworm larvae used in the experiments which will be described in this paper were taken from cultures five to eight days old, with the exception of those used in the series on the length of life of unsheathed larvae which were from an eighteen-day-old culture. The same technique was employed in counting and in preparing the soils as in the migration experiments previously described (Augustine, 1922*a*). Two sizes of containers were used, viz., (1) tin pudding pans, six inches in diameter and two inches in depth, and (2) three-ounce glass jars.

DATA ON LENGTH OF LIFE FROM MIGRATION EXPERIMENTS.

Early in my work on the migration of hookworm larvae in soils (Augustine, 1922*a*) I was struck by the great reduction in the numbers of larvae recovered from experiments which had run a few weeks. Table 2 gives the data on the reduction of hookworm larvae in ten of these experiments. Although the experiments were made under favorable conditions, after a period of twenty-one days, there occurred a reduction in the numbers of larvae of over 90 per cent. Stimulated by these findings, experiments were planned to further test this point.

TABLE 2.

Showing reduction in the numbers of hookworm larvae in the migration experiments.

No. Exp.	No. larvae placed in soil.	Type of soil.	Time larvae remained in soil.*	Larvae recovered.		
				Un-sheathed.	Sheathed.	Total.
1	1,000	Moist clay loam	7 days	141	180	321
2	498	Moist sand	10 days	81	10	91
3	512	Moist clay loam	11 days	234	3	237
4	1,100	Moist clay loam	21 days	11	7	18
5	392	Moist clay loam sod	21 days	1	4	5
6	500	Moist red clay loam	22 days	6	3	9
7	361	Moist clay loam sod	30 days	1	1	2
8	500	Moist clay loam	35 days	2	3	5
9	426	Moist clay loam	37 days	0	1	1
10	650	Moist clay loam	42 days	0	1	1

EXPERIMENTS ON LENGTH OF LIFE OF MATURE HOOKWORM LARVAE IN SOILS.

Table 3 gives data from a series of thirty experiments to determine the amount of mass reduction in a known number of mature hookworm larvae in three types of soil, viz., (1) clay loam, (2) sand, and (3) a clay loam soil, under favorable conditions of temperature and moisture. In these experiments the soil was placed to a depth of 1½ inches in thirty of the six-inch pans, ten pans with each type of soil. Three hundred active, sheathed hookworm larvae from cultures five and seven days old were added to each pan. The pans were then placed in a shady place and the soil kept moist throughout the experiment. Examinations, by the isolation apparatus, of the soil from one pan of each type were made weekly for seven weeks and the larvae recovered counted. As the seventh examination gave negative findings for hookworm larvae the eighth examination was made three days later. As this also proved to be negative for all three soils the experiment was closed. The results of these experiments (Table 3) indicate a rapid dying off of the larvae within the first two weeks of the experiment. If the time of development in the cultures is added, it is seen that in three weeks after the eggs left the host 90 per cent. of the larvae which developed to the infective stage had perished. The significant points brought out in this series (Table 3) are (1) the high reduction occurring within two or three weeks, (2) the fairly constant number of larvae remaining alive up to seven weeks, and (3) a complete dying

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* To give the total age of the larvae, at the conclusion of the experiment, the time of their development in the cultures (5 to 7 days) must be added to the time they remained in the soil.

TABLE 3.

Showing mass reduction of hookworm larvae in 3 types of soil.

In each of the experiments 300 sheathed hookworm larvae, taken from cultures 5 to 7 days old, were placed in 6-inch pudding pans containing 1½ inches of soil. Experiments started July 5, 1921.

Exp. No.	Date of examination.	Clay loam soil.				Sandy soil.				Clay loam soil.			
		Larvae recovered. Unsheathed.	Larvae recovered. Sheathed.	Total.	Per cent. reduction.	Larvae recovered. Unsheathed.	Larvae recovered. Sheathed.	Total.	Per cent. reduction.	Larvae recovered. Unsheathed.	Larvae recovered. Sheathed.	Total.	Per cent. reduction.
1	July 12	52	4	56	81.3	123	9	132	56	30	9	39	87
2	July 19	16	1	17	94.3	38	1	39	87	10	2	12	96
3	July 26	14	5	19	93.6	13	0	13	95.6	15	3	18	94
4	August 2	13	5	18	94	11	1	12	96	0	0	0	100
5	August 9	5	0	5	98.3	6	8	14	95.3	6	8	14	95.3
6	August 16	1	14	15	95	4	0	4	98.6	2	7	9	97
7	August 23	0	0	0	100	0	0	0	100	0	0	0	100
8	August 25	0	0	0	100	0	0	0	100	0	0	0	100
9 and 10	Not examined												

out after that time. It would therefore appear that the activities of the larvae are at first intense, which is followed almost immediately by a high death rate.

TABLE 4.

Showing mass reduction of hookworm larvae in a clay loam and sandy soil.

In all these experiments the 3-oz. glass jars were used, filled ¾ full with soil.

Exp. No.	All experiments started July 19.	Clay loam soil.					Sand.				
	Date of examination.	Number of larvae placed in soil.	Larvae recovered.		Total.	Per cent. reduction.	Number of larvae placed in soil.	Larvae recovered.		Total.	Per cent. reduction.
			Unsheathed.	Sheathed.				Unsheathed.	Sheathed.		
1	July 22	300	128	161	289	3.6	300	83	159	242	19.3
2	July 25	300	115	144	259	13.6	311	99	96	195	37.2
3	July 28	300	69	54	123	59.0	321	90	67	157	51.0
4	July 31	300	42	29	71	76.3	308	107	70	177	42.5
5	Aug. 3	300	59	29	88	70.6	303	29	9	38	87.4
6	Aug. 6	300	33	12	45	85.0	316	48	3	51	83.8
7	Aug. 9	300	40	7	47	84.3	304	40	15	55	81.9
8	Aug. 12	300	5	0	5	98.3	331	38	0	38	88.8
9	Aug. 15	300	13	5	18	94.0	325	26	1	27	91.6
10	Aug. 18	300	3	0	3	99.0	304	21	1	22	92.1

Table 4 gives the results of a series of experiments similar to those summarized in Table 3, except that the examinations were made every third day and the containers were the three-ounce glass jars. As the data show, the greatest reduction occurred about two weeks after the experiment was started, or about three weeks after the deposition of the eggs.

THE RELATION OF LOSS OF SHEATH TO LENGTH OF LIFE.

As has been previously stated (Cort, Augustine, et al., 1922) a large proportion of the mature hookworm larvae lose their sheaths in the soil. This discovery immediately introduces a new factor into the study of the length of life of the infective hookworm larvae. Three questions present themselves, viz., (1) Does the loss of the protective sheath directly shorten the life of the larvae? (2) Does the loss of sheath make the larvae more susceptible to unfavorable conditions of environment and thus shorten their life under certain conditions? and (3) Does the loss of sheath render the larvae non-infective, which would be the equivalent of terminating their lives?

TABLE 5.

On the mass reduction of hookworm larvae in loam soil from cane field.

Ten experiments started July 18, with 100 unsheathed larvae in each. All larvae were obtained from same culture, 18 days old.

Experiment number.	Date of examination.	Number of larvae recovered.	Per cent. reduction.
1	July 21	87	13
2	July 24	52	48
3	July 27	51	49
4	July 30	15	85
5	August 2	13	87
6	August 5	4	96
7	August 8	7	93
8	August 11	0	..
9	August 12	0	..
10	August 12	0	..

Table 5 gives the data on a series of experiments on the length of life of unsheathed hookworm larvae in clay loam soil under favorable conditions. The larvae used in these experiments were isolated from soil on which a stool containing hookworm eggs had been placed eighteen days before. Of the 1,300 larvae obtained only 10 were found enclosed in sheaths. The experiments were made under the same environment as those with the sheathed forms. Examinations were made every third day as long as the results were positive. After the first negative appeared, which was 24 days after the experiments were started, or 42 days after the stool had been placed on the soil, the remaining jars were examined on the day following. These were likewise negative. While this series of experiments indicates that the life of the unsheathed hookworm larvae may be similar to that of the sheathed forms it must be taken into consideration that there undoubtedly had occurred a great reduction in the numbers of larvae before isolating them from the culture, and that the larvae used in the experiment were the survivors of this reduction.

While no experiments were made to test the comparative resistance of the unsheathed and the sheathed larvae, it was found that upon the addition of a few drops of dilute formalin (See Cort, Ackert, et al., 1922, p. 5) to the preparation before counting, the unsheathed larvae quickly succumbed, while the sheathed forms were stimulated to greater activity, and could resist the formalin for a considerable time. This observation indicates, as would be naturally inferred, that unsheathed larvae are less resistant to unfavorable conditions than sheathed. This would then lead to a more rapid dying of the unsheathed larvae whenever conditions became unfavorable. Since our present knowledge of the resistance of the mature hookworm larvae to various conditions is based entirely upon studies of the sheathed forms it, will be necessary to repeat much of this work with the unsheathed larvae, to determine what actually happens under natural conditions.

That sheathed hookworm larvae complete their second ecdysis, while penetrating the skin, and become adults in the intestines of man has been conclusively demonstrated by a number of investigators. Whether the loosing of the sheath in the soil renders the larvae non-infective, as far as man is concerned, which would be equivalent to their death, is not yet known. To gain information on this point unsheathed larvae were applied to the shaven belly of a male white rat, sixteen months old. Upon examination with a binocular microscope the larvae were seen to quickly spread themselves over the surface of the skin as far as the moisture extended. As the moisture around the larvae evaporated penetrating movements were started. The anterior ends of the worms would be lifted into the air and then turned toward the skin again, touching here and there as if seeking some port of entry. In one particular instance a larva was seen to leave the shaven area, to settle at the base of a hair and push one third of its body into the hair follicle. Then after a moment's rest the posterior end swung up parallel with the hair and with several slow twists the worm moved further into the skin and again rested. This movement was repeated several times until the tail of the worm vanished beneath the surface of the skin. Two other unsheathed larvae were seen to penetrate the skin within the shaven area. The entire process took less than a half hour. One hour later water was applied to the infected spot and upon microscopical examination no larvae were found. While this observation only shows that unsheathed hookworm larvae can penetrate mammalian skin, it seems very probable that they can also reach the intestine and grow to maturity. Further experiments are needed to clear up this point.

Discussion of results.

This experimental evidence on the length of life of the infective hookworm larvae in the soil is supported by the field observations of Cort and Payne (1922*a*, p. 138). In one instance soil pollution was found near the edge of a cane field and soil samples from this area showed it to be an intense center of infestation. This area was visited frequently at later intervals, but no further evidence of soil pollution was found. Soil samples, taken three weeks later from the same place, yielded but nine hookworm larvae, and later examinations showed a complete dying out of the larvae. More striking still is their study of the mass reduction of hookworm larvae in a chosen area of this field which, before educational work, sanitation, and treatment were started, was found to have a very heavy infestation with hookworm larvae. In less than three weeks after the reduction of soil pollution, which immediately followed the introduction of control measures, a second series of examinations was made. The results of these examinations showed a reduction of over 90 per cent. from the first series. Subsequent examinations of samples taken from this area showed still further reductions. In about six weeks after pollution of the soil had been stopped this once heavily infested strip of cane field had become practically free from hookworm larvae. Similar results were obtained during their studies on the sources of hookworm infestation in a cacao grove (Cort and Payne, 1922*b*). Although soil pollution was not checked samples of soil taken from grossly polluted areas, six weeks after treatment, showed a marked reduction in the number of infective hookworm larvae present.

The results of these laboratory experiments and field observations show that the life of the hookworm larvae in the soils of Trinidad is limited to a few weeks. Whether this finding is constant under a variety of conditions is not known, and experiments are planned to test this point. These results are very different from those obtained with sheathed larvae under laboratory conditions at lower temperatures. It would seem that the differences in these results can be explained by differences in the conditions of the experiments. The conditions of the experiments of the early workers (see Table 1), in which larvae were kept alive for such long periods, were such that the activities of the larvae were greatly reduced and the protective sheaths retained. In my experiments in Trinidad the daily range of temperature during the course of the work was from about 74° F. to 94° F., a temperature which, if other conditions were favorable, tended to keep the larvae active. Further it was found that a large proportion of the larvae lost their sheaths in the soil. While it is not possible from the information at hand to find any direct correlation between the process of unsheathing and the death rate under favorable conditions, it was found that the unsheathed larvae are more susceptible when in unfavorable environments. As the unsheathing of the larvae appears to be a continuous process, as long as the soil remains infective, the newly unsheathed larvae would be constantly killed when becoming exposed to diverse conditions, even though sheathed larvae were able to withstand the diversity. It can be concluded, therefore, that conditions favorable to intense activity and to the loss of the sheath will tend to shorten the life of mature hookworm larvae in the soil.

SUMMARY.

1. From the observations of various investigators on hookworm larvae under unnatural conditions the opinion has become current that they live for months or even years in the soil, under favorable conditions of temperature and moisture.

2. Laboratory experiments carried on in Trinidad, British West Indies, from May to September, 1921, show that a rapid reduction in the number of hookworm larvae occurs in soils of various types, and that the extent of their life is limited to about six weeks.

3. The completion of the second ecdysis of the infective hookworm larvae in the soil presents a new factor in determining their length of life, which was not found to directly shorten the life of the larvae under favorable conditions, but renders them more susceptible when in unfavorable environments.

4. Experimental evidence indicates that the loss of sheath does not render the mature larvae non-infective which would be the equivalent of their death.

5. The conclusion can be drawn that environmental conditions, such as tropical temperatures which tend to increase the activity of the mature hookworm larvae, will shorten their lives by the more rapid using up of the stored food material.

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THE HUMAN INTESTINAL AMOEBA, IODAMOEBA WILLIAMSI, AND ITS CYSTS (IODINE CYSTS).*

By W. H. TALIAFERRO, Ph.D., and ELERY R. BECKER, A.B.

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Plates VIII and IX.

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Introduction.

The "iodine cysts" which Wenyon originally described as occurring in human faeces have now been found by a number of investigators. Not only have they been found in human faeces, but O'Connor (1920) and Chauchemez (1921) have noted the presence of an "iodine cyst" in the intestinal contents of the pig which is indistinguishable from the human form. These peculiar bodies differ markedly from the common intestinal cysts of man. While a large number of them are spherical in shape, some present very bizarre and irregular outlines, and in freshly passed cysts the cytoplasm contains a large inclusion of glycogen which takes the typical mahogany-brown stain when observed in iodine. The mature cysts are typically uninucleate, and, as we will see later, the structure of this nucleus is different from that of the other intestinal cysts.

While there is no doubt at the present time that the "iodine cysts" represent the encysted stage of an amoeba, there is considerable variance of opinion as to, first, the systematic relationship of this amoeba, and second, the structure of the nucleus, both in the motile amoeba and in the cysts. The object of the present investigation was to make a careful study of both the motile and encysted forms, with especial reference to the structure of the nucleus. With this background we hope to be able to correlate better the findings of different investigators, and to discuss the nomenclature of the species. The most interesting thing in the present work is that, although our findings support the contentions of Dobell (1919), we have been able to duplicate the findings of other investigators by using different microscopical methods.

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* From the Department of Medical Zoology of the School of Hygiene and Public Health of the Johns Hopkins University.

Historical review.

As stated above, these cysts were first observed in human faeces by Wenyon (1915) who described them as " spherical bodies " with an " iodophilic " inclusion. A year later the same author redescribed them under the name of " iodine cysts " or " I. cysts." In his first paper he suggested that they were probably vegetable in nature, and in the second paper he believed that he had seen them germinate when kept in faeces. In a still later paper Wenyon and O'Connor (1917) state that besides the typical " iodine cysts " there are sometimes present larger spherical bodies which appear homogenous, with a central vacuole, which, they believed, probably " represent large I-cysts which are devoid of iodophilic bodies or structures of an allied nature."

Kuenen and Swellengrebel (1917) found the " iodine cysts " and an active amoeba, which very probably was the active amoeboid form of the cysts. They provisionally named this active amoeba, " Pseudolimax."

Matthews (1918) again described and figured " iodine cysts." This investigator followed Wenyon's suggestion that they were probably of a vegetable nature.

Flu (1918), in the same year, came to the conclusion that these cysts were, in reality, degenerate cysts of *E. tetragena* (*Endamoeba histolytica*).

After reconsidering the whole matter, Brug (1919) came to the conclusion that the " iodine cysts " are the encysted stage of an amoeba described by Prowazek (1911) under the name, *Entamoeba williamsi.* He was also of the opinion that this amoeba should be placed, with *E. nana,* in the genus *Endolimax* Kuenen and Swellengrebel, 1917. In this paper Brug gives a description of the nucleus of the motile amoeba as well as the cysts. The noteworthy feature of this description is that he described, in the nucleus of the cysts, besides a large eccentric karyosome, a crescent-shaped mass of chromatin granules. We will take up a discussion of this description later.

In the same year Kofoid, Kornhauser and Swezy (1919) came to the conclusion that Wenyon and O'Connor's "iodine cysts" include two different organisms. They came to the conclusion that the homogeneous bodies seen by these investigators and considered by them to be "iodine cysts" devoid of their iodophilic inclusions are "the non-nucleated, homogeneous spore-like cysts of some vegetable organism, possibly the chlamydospore of some phycomycete of unknown affinities," and that the large glycogen-laden "iodine cysts" are uninucleate cysts of *Endolimax nana*. It is not necessary to consider these authors' conclusions in regard to the homogeneous bodies included by Wenyon and O'Connor in the "iodine cysts," because we feel that these bodies are probably not typical "iodine cysts." It is with the second conclusion that we are most interested, namely, that the "iodine cysts" are the uninucleate cysts of *Endolimax nana*. As we understand it, these authors base this conclusion on the following points: In the first place, it is known that the common intestinal amoebae (*E. histolytica, E. coli,* and *E. nana*) often have large glycogen masses during the early one- and two-nucleate stages of their cysts, and if the "iodine cysts" are, in reality, uninucleate cysts of *E. nana,* it is not surprising to find a large glycogen mass in them. In the second place, they maintain that the nucleus of the "iodine cysts" is similar to that of an uninucleate *E. nana* cyst in respect to: (*a*) dimension of nucleus, (*b*) position of nucleus in cyst, (*c*) structure, both as regards nuclear membrane, and arrangement and distribution of the chromatin. And in the third place, they maintain that there is an intergradation in size of the glycogen mass found in the "iodine cysts" and in uninucleate cysts of *E. nana*. Besides the question of the structure of the nucleus, which will be discussed in detail in a later section, there are two difficulties in the way of accepting the conclusion of these authors. The first of these is that the "iodine cysts" are, on the average, larger than those of *E. nana;* and the second is that these authors were unable to find any of the larger cysts with four nuclei which should occur, if these were cysts of *E. nana*. As a matter of fact, the great majority of the "iodine cysts" are uninucleate, and it is only rarely that a specimen with even two nuclei is seen. That these difficulties were clearly realized by these investigators is shown by the following quotation: "In fact two nuclei are found in the larger cysts and, as yet, we have not found four in them. It seems probable that there may be a decrease in the size of the cyst, as the glycogen decreases in volume and as the nuclei increase from two to four." It is very difficult to accept this latter suggestion, because we have no evidence that once an amoeba secretes a cyst wall about itself that this cyst wall ever diminishes in size. Certainly in the case of *E. coli* we do not find any decrease in the size of the cyst as the organism uses up its mass of glycogen. This is true, although the binucleate cyst of *E. coli* often contains an enormous mass of glycogen.

Dobell (1919), working independently and without the knowledge of Kofoid, Kornhauser and Swezy's work, came to a conclusion which is radically different from these investigators. He maintained that the "iodine cysts" are the cysts of an amoeba which was first described by Prowazek under the name of *Entamoeba bütschlii.* This is also the same amoeba which Kuenen and Swellengrebel (1917) provisionally named "Pseudolimax." As this name was not proposed according to the rules of nomenclature, and as it was clearly not an *Entamoeba,* Dobell proposed the name, *Iodamoeba,* for the genus. According to Dobell the nucleus of the motile amoebae possesses a large central karyosome which is surrounded by a layer of granules—the so-called "peripheral chromatin." These granules take both the nuclear and plasma stains, although they give up such stains as haematoxylin much quicker than the karyosome. In such haematoxylin stains they can be overstained, in which case they are often confused with the karyosome, or they can be completely decolorized, in which case they become invisible. Outside of this layer there is a well-defined nuclear membrane which may have granules imbedded in it. According to Dobell, when the amoeba encysts, the nucleus undergoes a remarkable change, in that the karyosome migrates to the edge of the nuclear cavity and the remaining space becomes filled with large granules, such as were originally arranged in a single layer around the karyosome.

Although Dobell's work seems to be a very thorough and painstaking study, his conclusions are not accepted by some of the recent authors. Thus, in a description of a new amoeba from the human intestine (*Councilmania lafleuri*), Kofoid and Swezy (1921) reiterate their former view that the "iodine cysts" are cysts of *Endolimax nana.* They state that they are unable, with their methods of fixation and available stains, to demonstrate in the nucleus of *C. lafleuri*, the eosinophilic granules which Dobell describes for the amoeba of the "iodine cysts." They even suggest that, with this exception, there is enough in common with their description of *C. lafleuri* and Dobell's description of *Iodamoeba* to make it desirable to reinvestigate Dobell's *Iodamoeba* to see whether or not it should not be considered *C. lafleuri*. It is, however, a little difficult to see their grounds for this suggestion, as the range of size of the two species is not at all similar—*Councilmania* being a very large amoeba and *Iodamoeba* being a very small form.

Brug (1921) has also returned to the study of the amoeba of the "iodine cysts." His description of the amoeba and its cysts is essentially the same as that given in his earlier paper. He also still feels that the amoeba should be called *Endolimax williamsi* Prowazek, 1911. In regard to the nomenclature he has one very interesting new fact to offer, in that he has been able to examine Prowazek's original slides of "Entamoeba williamsi." He finds that these slides contain both *E. coli* and the amoeba of the "iodine cysts." He feels, therefore, that although *E. bütschlii* Prowazek, 1912, is entirely the amoeba of the "iodine cysts," *E. williamsi* Prowazek, 1911, is in part the same species, and that the latter name should have precedence over the former.

Nöller (1921) has been able not only to examine Prowazek's original slides of *E. williamsi*, but also of *E. bütschlii*. This examination supports the contentions of Brug. Nöller feels, however, that since Prowazek's description of *bütschlii* is based solely on the amoeba of the "iodine cysts," whereas the description of *williamsi* is based only in part on this amoeba, the sensible thing is to accept the former name. He therefore uses the name, *Iodamoeba bütschlii* Prowazek, 1912, emend. Dobell, 1919.

Besides the work cited above, Rodenhuis (1919) has proposed the name *Endolimax pileonucleatus* for the amoeba in question. The author's grounds for proposing this name are not clear. One thing of interest in this paper is that the author has found and described what he believes to be several stages in the division of the nucleus. In the paper in which Chauchemez (1921) describes the finding of "iodine cysts" in the faeces of pigs, he notes that Brumpt (personal communication) accepts Dobell's genus *Iodamoeba*, but because he does not believe that the amoeba can be identified with any previously described species, he (Brumpt) proposes the name *Iodamoeba wenyoni*.

Material and methods.

While a number of stools containing "iodine cysts" have come to our notice during the last two years, practically all of the present work was carried out on specimens obtained from two individuals. These individuals were selected because we could obtain daily samples of faeces from them, and no objection was raised whenever we wished to administer a purgative in order to obtain the motile amoebae. They are sisters, aged three and five years, who were born in Baltimore and have not left the state of Maryland during their life time. We have designated them Cases 1 and 2 respectively. As far as we can find out, they are very healthy children who have no history of intestinal disorder. They show a rather heavy multiple infection of intestinal protozoa, having, besides the amoeba of the "iodine cysts," *Endamoeba coli, Trichomonas hominis,* and *Giardia intestinalis.* Besides these entozoa with which both cases are infected, Case 1 is infected with *Dientamoeba fragilis.* Neither case is infected with *Endolimax nana.*

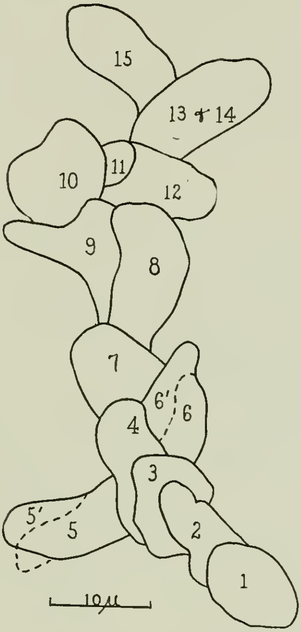
In addition to studying the organisms in the living condition, they were fixed and stained in various solutions. Most of our material was fixed in Schaudinn's sublimate-alcohol solution, to which were added varying amounts of acetic acid, and then stained either with Heidenhain's iron haematoxylin, followed with eosin, or with Mann's methylblue-eosin stain, as modified by Dobell. A large amount of material was, however, fixed and stained in other ways in order to study it from a comparative standpoint. Among these latter fixatives may be mentioned: Carney's acetic acid-alcohol-chloroform mixture, Fleming's chromo-acetic-osmic mixture (strong formula), Bouin's picro-formol mixture, and chromo-aceto-formalin mixture. The other stains that were used were Delafield's haematoxylin and Dobell's alcoholic iron-haematein.

It is a pleasure to ackowledge our indebtedness to our laboratory assistant, Mr. Conrad Bauer, for finding the cases described above, and for obtaining faecal specimens from them daily over a period of several months. We are also indebted to Miss Ethel Norris for the drawings which appear in this paper.

The motile amoeba.

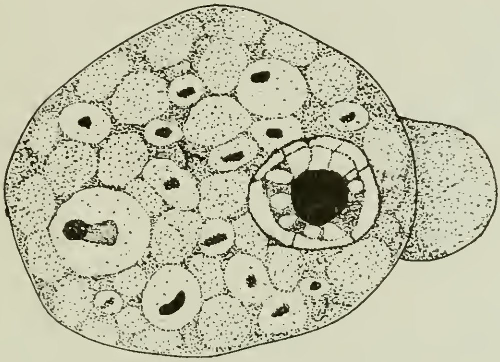
In the present paper we will limit ourselves to a detailed description of the nucleus of the present form. There are, however, certain other characteristics of the species which should be mentioned. According to Dobell (1919) the diameter of this amoeba, when rounded, is usually from 9 to 13 μ. He also observed larger specimens up to 17–20 μ and smaller ones of 5 μ in diameter. Kuenen and Swellengrebel (1917) give the diameter as 10–12 μ and Brug (1921) states that in his experience it is 7 to 20 μ. The maximum size which Prowazek (1912) gives for his *Entamoeba bütschlii,* which is probably the same species, is 24 μ. As Dobell (1919) points out, however, Prowazek may have mistaken a small specimen of *E. coli* for the present form. In our work we find that fixed and stained specimens of the motile amoeba, when rounded, vary in diameter from 9 to 14 μ, the most usual diameter being 11 μ. The largest specimen which we have seen was 20 by 15 μ. In this connection it is interesting to note that, in our experience, the active amoebae are on the average about 2 μ greater in diameter than the cysts. This indicates that the amoeba undergoes a reduction in size just before encystment. Such a process is known to occur in the other intestinal amoebae, but Dobell (1919) maintains that it is not the case in the amoeba of the "iodine cysts."

When the motile amoebae are examined in the living condition on a warm stage, their movement is very similar to that of a small *E. coli.* The rapidity with which different specimens move varies greatly, some being very sluggish and others moving with great rapidity. Text-figure 1 represents a series of camera lucida outlines of a rapidly moving specimen drawn at intervals of 15 seconds. In such specimens there is usually no sharp distinction between ectoplasm and endoplasm. Occasionally, however, a specimen will stop its progressive movement and send out a pseudopodium which is sharply marked off from the granular body of the animal. These clear hyaline pseudopodia are formed very rarely, and we have never seen them used as organs of locomotion. We believe that they represent an abnormal condition, and that the formation of the *coli*-type of pseudopodium is the normal method of progression. In contrast to such active specimens as the one shown in Text-fig. 1, we have often observed specimens for as much as 15 minutes which constantly threw out pseudopodia and yet scarcely moved from their original position. The visibility of the nucleus in the living amoebae depends largely on the amount of the food inclusions. Specimens are often seen which contain numerous bacteria and almost every type of intestinal debris, as in the specimen shown in Fig. 1, Plate VIII. Such specimens, when alive, remind one of *E. coli,* but the nucleus is rarely visible in them. In other specimens which contain little or no food, the nucleus with its large central karyosome can often be clearly seen.



Text-fig. 1. Series of camera lucida outlines of a very active specimen of *Iodamoeba williamsi* which shows the characteristic changes in shape in such specimens. The specimen was observed on a warm stage, and the outlines were made at 15 second intervals. Numbers 1–15 indicate the shape and position of the amoeba at each consecutive interval.

The size of the nucleus varies with that of the amoeba, being roughly about one quarter the diameter of the entire specimen. Its structure can be considered under four heads: (1) the nuclear membrane, (2) the karyosome, (3) the peripheral layer of granules (" peripheral chromatin "), (4) the linin threads which extend from the karyosome to the nuclear membrane. The nuclear membrane is clearly visible in any of the stains used and often contains granules or even small lumps of deeply staining substance. Whether or not these masses are chromatin cannot be said at the present time. The large karyosome is, in our experience, always located centrally in the nuclei of the motile forms. Its diameter is roughly about one half that of the nucleus (see Plate VIII, Figs. 1–4). When stained with haematoxylin stains, it often is not homogeneous in structure, but exhibits a lighter central area. When stained with Mann's stain, there are often present one or several darker staining granules in the center. Figure 3, Plate VIII, shows a specimen which contains three of these structures. The nature of these structures is not clear—certainly there is no valid reason for assuming them to be centrioles, as is done by Prowazek (1912) in his description of "Entamoeba bütschlii," which is probably the same form. Immediately surrounding the karyosome, there is a layer of granules which we have termed the peripheral granules and which is often termed "peripheral chromatin." In a large number of the specimens stained with either iron haematoxylin and eosin, or with Mann's stain, this layer of peripheral granules appears as a simple layer divided into segments by numerous radiating septa (Plate VIII, Figs. 1 and 2). In well-stained specimens, however, it can be seen that this is not the true structure of this layer, but that each segment is in reality an ovoid granule embedded in a deeper staining matrix. (Plate VIII, Figs. 3 and 4.) In the most favorable specimens, with careful focusing of the microscope it is even possible to see that each of these granules has a slightly higher refractive index than the surrounding protoplasm. This layer of peripheral granules is sharply differentiated from the karyosome, in material stained in Mann's stain—the layer of granules staining blue and the karyosome red. The appearance in iron haematoxylin and eosin depends largely on the extraction of the haematoxylin. As pointed out by Dobell (1919) the granules take both nuclear and plasma stains, but give up the former much more readily than the karyosome. Not only is this true in our experience, but we have also found that the matrix in which the granules are embedded is intermediate in this respect—decolorizing much sooner than the karyosome, but retaining the stain (haematoxylin) longer than the actual granules It is, therefore, possible to stain the entire granule layer black like the karyosome, or to decolorize the nucleus until the actual granules are colorless, but the matrix around them is still dark (Plate VIII, Fig. 3), or to decolorize the nucleus to such an extent that the entire granule layer—including both the actual granules and their surrounding matrix—appears practically colorless, with the karyosome still retaining an almost black stain. In the latter case the entire granule layer can be restained with eosin. While the above description of the way in which the granules behave with haematoxylin stains is true in general, one finds that each granule may show some idiosyncrasy in the way in which it retains the stain. Such a condition of affairs is shown in the nucleus of the specimen in Text-fig. 2. Here some of the granules have completely given up the stain, while others cannot be distinguished from the matrix in which they are embedded. We shall see later that this variability with which the different granules take or give up haematoxylin probably explains some of the peculiar pictures which are often seen in the nuclei of cysts.



Text-fig. 2. Camera lucida drawing of a motile specimen of *I. williamsi*, stained with iron-haematoxylin. Note the peculiar way in which some of the peripheral granules around the karyosome are stained while others are not. Compare with Figs. 1–4, plate VIII. × 4,300.

In the majority of our specimens fixed with Schaudinn's fluid and stained with either iron haematoxylin and eosin or with Mann's stain, it is possible to distinguish several fine linin strands running from the karyosome to the nuclear membrane. These linin fibers must not be confused with the appearance of septa in the granule layer noted above. These linin fibers extend through the granule layer and actually touch the nuclear membrane (Plate VIII, Figs. 1–4). Dobell (1919) does not mention these strands, but they are noted by Brug (1921). It is very difficult, however, to interpret Brug's figures. We are unable from a study of his figures of the motile form to decide whether he has decolorized the layer of peripheral granules to such an extent that a few "septa" are left, or whether he has overstained them so that the layer of granules is not differentiated from the karyosome, in which case the strands shown would be the linin strands in question. Judging from the comparative size of the karyosome to the entire nucleus, we feel that the latter is probably the true explanation of his figures.

differences between the foregoing account and the figures given by Brug (1921) are to be explained by an overstaining of his specimens with haematoxylin. In such overstained specimens, the layer of peripheral granules cannot be differentiated from the karyosome, and this gives the nucleus the appearance of containing a large central karyosome, connected to the nuclear membrane by a few linin strands.

THE CYST.

It is difficult to give a single measurement of size for the cysts, due to the fact that so many of them are irregular in shape. To overcome this difficulty in the case of the irregular cysts, Dobell (1919) has taken the average of the greatest length and the greatest width, as the " size." In using this measure of " size " which we have found very valuable, we have found that most cysts lie between 6.4 μ and 16.6 μ, and that the average is about 9.1 μ. In our experience, the irregularity in shape of the cysts varies considerably from day to day in the same infection. Some days almost all of the cysts are nearly spherical, and on other days many are very irregular in shape. In this connection mention should be made of a most remarkable structure which we found on one of our prepared slides, which is undoubtedly an " iodine cyst." A camera lucida outline of this cyst is given in Text-fig. 3 *B*. By comparing this cyst with the one beside it, which is of ordinary size, it can be seen that it is abnormally large and irregular in outline. This monster contained four glycogen vacuoles. In spite of its abnormalities, however, the structure of the nucleus leaves no doubt that it is a true " iodine cyst." In our work we have not found any other cysts which were comparable to this one, either in size or in shape.

It is not necessary to describe the appearance of the cysts, when examined in the living state, or in iodine solution, as this has been done by a number of investigators. We can, therefore, pass directly to a consideration of the nuclei of the cysts. The mature cysts practically all contain a single nucleus. Occasionally supernucleate cysts, with two nuclei, are found, but these are rare. In order to ascertain approximately the percentage of the cysts that are binucleate, we examined 2,000 cysts consecutively, as they appeared on some of our prepared slides. Among those 2,000 cysts only four contained two nuclei—giving a percentage of 0.2. As far as we can find, no investigator has described cysts with more than two nuclei.

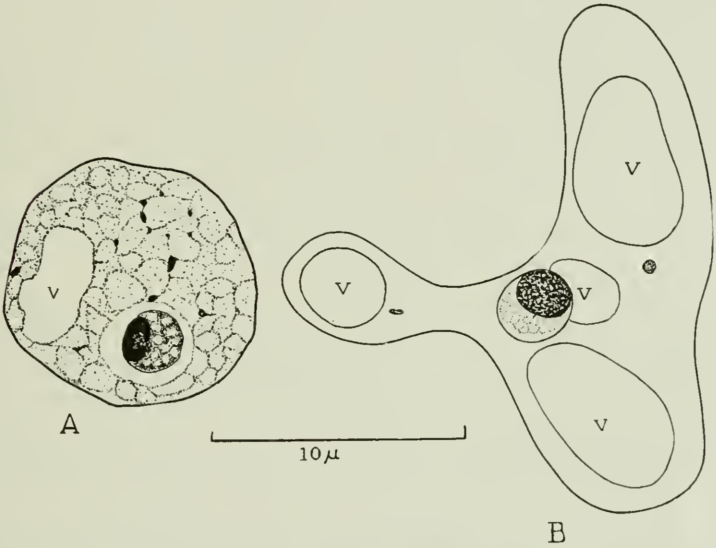
With the exception of the presence of the fine linin fibers extending

ERRATUM

The top line of page 198 should be preceded by the paragraph beginning at the bottom of this page and ending with the third line of page 199.

from the karyosome though the layer of peripheral granules to the nuclear membrane, the foregoing account agrees, almost in detail, with the description given by Dobell (1919). We believe further that the

The nucleus of the cyst possesses the same structures which were described for that of the motile form, viz., (1) a nuclear membrane, (2) a karyosome, (3) peripheral granules ("peripheral chromatin"), and (4) linin strands running from the karyosome to the nuclear membrane. Structures (1) and (4) are the same as in the motile amoebae, but the position and relationship of (2) and (3) are greatly modified, when the amoeba encysts. These changes can readily be seen by comparing the nuclei of the motile forms, shown in plate VIII (Figs. 1–4), with those of the cysts, shown in the same plate (Figs. 5 and 6). The karyosome, instead of being central, as it is in the motile amoeba, becomes eccentrically placed, and the layer of granules, instead of surrounding the karyosome, becomes massed to one side of it. The number of these granules varies greatly. Sometimes they are several layers deep and completely fill the nuclear cavity on one side of the karyosome, and at other times, they are comparatively few in number and are closely applied to one side of the karyosome (Plate VIII, Figs. 5 and 6). Of course the actual appearance of the nucleus



Text-fig. 3. A, typical cyst of *I. williamsi* with a single glycogen vacuole, *v*. B, unusually large and irregular cyst containing four glycogen vacuoles, *v*.

varies according to the pole from which it is viewed. If it is seen from the pole toward which the granules are displaced, the contour of the karyosome is obscured, giving a picture like the one shown in Plate VIII, Fig. 7. If, on the other hand, it is seen from the opposite pole, i.e., the pole toward which the karyosome is displaced, the latter is seen surrounded by the granules (Plate VIII, Fig. 8). In comparing these two figures (Plate VIII, Figs. 7 and 8), it must be kept in mind that they differ markedly in another respect, viz., the number of granules present in each.

As in the case of the motile amoebae, our description of the structure of the nucleus of the cysts agrees, almost in detail, with that of Dobell (1919). Dobell, however, does not note or figure the linin strands, running from the karyosome to the nuclear membrane, which are shown in figure 6, and, to a less extent, in Fig. 5, Plate VIII. Most of our material, which was fixed in Schaudinn's fluid and stained in either iron haematoxylin and eosin or in Mann's stain, showed these strands. They were, however, absent in some of the cysts, as is shown in Text-fig. 3 *A*. Incidentally this figure is very similar to some of those given by the above investigator.

While our results are in accord with those of Dobell, they are not in agreement with those of Brug (1919 and 1921), Nöller (1921), or Kofoid, Kornhauser, and Swezy (1919). Let us first consider the findings of Brug. In his earlier paper (1919) he describes the nucleus as containing a large karyosome and a crescent-shaped mass of chromatin granules which is separated from the karyosome by a considerable space. This description is repeated in his later paper (1921). It is very difficult to ascertain the exact nature of this crescent-shaped mass of chromatin granules from his description. One would be led to think that the author means that the crescent-shaped mass is composed of chromatin of the same nature as found in the karyosome. Certainly, there is no evidence that he means anything like the peripheral granules which we have just described, and which show an entirely different staining reaction from the karyosome when stained with Mann's stain. This interpretation is further supported by the fact that the author places the amoeba in the genus *Endolimax*—presumably on his interpretation of the structure of the nucleus. Another thing of interest is that the author apparently believes that the crescent-shaped mass of chromatin granules arises only after the amoeba encysts, whereas the peripheral granules, which we have just described, were represented in both the free forms and in the cysts.

We were, at first, unable to explain Brug's figures. Later on in our work, however, we obtained preparations which appear so similar to his that we feel that we have an explanation of his findings. In some of our material which was fixed with Schaudinn's fluid and stained with iron haematoxylin, we obtained many specimens similar to those shown in Plate IX, Figs. 9 and 10. These specimens show the presence of a crescent-shaped mass widely separated from the karyosome, and are almost identical with some of Brug's figures. One thing is at once apparent, however, viz., the crescent-shaped mass is composed of the same large granules ("peripheral chromatin") which Dobell and the present authors have described, as occurring between the karyosome and the nuclear membrane. The mass itself often stains as black as the karyosome, due to the way in which the matrix around the granules is stained. Why, then, should this mass of granules be so widely separated from the karyosome in this material, and not in the material described above? We first thought that it was because of shrinkage due to improper fixation. This is hardly the case, however, because in another set of the same slides, fixed in the same manner at the same time, but stained in Mann's stain, we could trace the granules clear up and in contact with the karyosome. It seems more probable, therefore, that the appearance of the crescent-shaped mass is to be explained on the assumption that the layer of granules next to the karyosome often gives up the haematoxylin stain more readily than the remaining granules. This assumption is supported by the fact that in haematoxylin specimens one can often see the granules extending to the karyosome, but those nearest to the karyosome are much more lightly stained than those further away. Another fact may enter into the explanation, and that is the question of the matrix in which the granules are embedded. This matrix undoubtedly takes the haematoxylin more readily than the actual granules, and the appearance of the separate mass may be due in some part to the presence of less matrix near the karyosome than further away. At any rate it is interesting to be able to duplicate Brug's results and see that his crescent-shaped mass is composed of the same peripheral granules which we described above. Furthermore, we feel that it is probable that in such specimens, as are shown in Plate IX, Figs. 9 and 10, there is a layer of unstained granules between the karyosome and the dark crescent-shaped mass.

While Nöller (1921) does not give a detailed description of the nucleus, it is clear that he agrees with the description given by Brug.

He states in a footnote (page 41) that the characteristic nuclear structure can best be seen in Delafield's haematoxylin or in haemalum preparations, and that the half-moon (crescent-shaped mass) can be seen only poorly in iron-haematoxylin preparations. He also states that not a single one of Dobell's figures depicts the true nuclear structure of the cysts, due to the fact that differentiation has been carried too far. In regard to Nöller's first statement we can say that we have been able to get this structure in iron haematoxylin as well as in Delafield's haematoxylin. We also believe that the second statement is based on a misinterpretation of the facts. It implies that the crescent-shaped mass is something different from the granules described by Dobell. As we have shown above, however, it is in reality composed of the same granules which Dobell shows in his figures, and which we have illustrated in Plate VIII, Figs. 5 and 6. Furthermore, we feel that the mass of these granules extends to the karyosome, and that there should be no space between them and the karyosome.

Besides the appearance of the crescent-shaped mass, many other peculiar nuclear pictures are often seen in iron-haematoxylin preparations. Some of these suggest very strongly the condition of affairs often met with in *E. nana*, and may offer an explanation of the results obtained by Kofoid, Kornhauser and Swezy (1919)—especially as these investigators used this stain almost exclusively. A few of these nuclei are shown in Plate IX, Figs. 11 to 16. In the case of the nucleus shown in Fig. 11, the mass of granules has been so poorly stained that the contour of none of them can be seen. Incidentally it may be remarked that this nucleus is viewed obliquely from the pole toward which the karyosome is displaced. In Figs. 12 and 14 a few of the granules themselves have apparently taken the haematoxylin stain. In Fig. 15 practically all trace of the mass of granules has been removed by destaining. The nucleus in Fig. 16 is very similar to that of *E. nana*. Here it seems probable that the larger black mass is the karyosome and the smaller one with the strand which attaches it to the larger mass represents the mass of granules. We do not know why some granules in the mass should decolorize so readily and others retain the stain, but we do know that the different granules often show many idiosyncrasies, in the way in which they behave, in the iron-haematoxylin process. We feel that, if any of these cysts had been stained in Mann's stain, we would have been able to demonstrate the karyosome and the peripheral granules, the former staining red and the latter blue, because some of the identical material, fixed at the same time, was stained in Mann's stain and did give these results. The above statements must not be construed to mean that accurate pictures of the nucleus are *never* obtained with iron haematoxylin. This stain, especially if used with eosin, often gives beautiful results, but it also often behaves as described above.

Our suggestion that the results of Kofoid, Kornhauser and Swezy can be explained in the above manner is not based on work with the " iodine cysts " alone. We have also examined a large number of both motile and encysted forms of *E. nana.* This was done to see if, with our technique, we could demonstrate the same type of nucleus in *E. nana* as we did in the " iodine cysts." In none of these specimens, however, have we been able to demonstrate anything comparable to the peripheral granules described above. The karyosome was always represented by one or several masses which were generally connected by a fine chromatic strand. A typical cyst of *E. nana* is shown in Plate IX, Fig. 17. All of our work, therefore, points to the conclusion that not only is the amoeba of the "iodine cysts" not identical with *E. nana*, but its nucleus is so different from that of the latter species, which is the type species of the genus *Endolimax*, that it cannot even be placed in the same genus.

Nomenclature[*]

The nomenclature of the parasitic amoebae is somewhat confused at the present time. The correct parent genus is undoubtedly *Endamoeba* Leidy 1879, type by original designation *E. blattae.* Since the formation of this genus the parasitic amoebae have been divided into a number of genera, the distinguishing characteristics of which are chiefly differences in nuclear structure (see Dobell and O'Connor, 1921). If we accept this classification the amoeba of the " iodine cysts " cannot be placed in the genus *Endamoeba* Leidy 1879, type *E. blattae* (*Entamoeba* Casagrandi and Barbagallo, 1895, type *E. coli*), or *Endolimax* Kuenen and Swellengrebel 1917, type *E. nana.* We believe that Brug's contention that the amoeba is a species of *Endolimax* rests on a misinterpretation of the nature of the crescent-shaped mass. If this mass were composed of the same type of chromatin as the karyosome, the nucleus would resemble an *Endolimax* nucleus. This is not the case, however, because the crescent-shaped mass is, in reality, an aggregation of the same type of large granules which surround the karyosome in the motile stage. While these granules may be a kind of chromatin, they do not give the same staining reaction as the karyosome, and are probably of an entirely different nature. The contention of Kofoid, Kornhauser and Swezy that the "iodine cysts" are the uninucleate cysts of *E. nana* has already been considered. As the amoeba cannot be placed in the genus *Endolimax*, the first available name is *Iodamoeba* of Dobell (1919). Kuenen and Swellengrebel's (1917) name, "Pseudolimax," was proposed, distinctly as not subject to the rules of nomenclature, and hence not as a true generic name.

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[*] We arrived at the conclusions expressed in this section, particularly those in regard to the specific name, after a conference with Dr. C. W. Stiles. It is a great pleasure to express our indebtedness to Dr. Stiles for his helpful advice.

Nöller (1921) and Brug (1921) have both examined Prowazek's original slides and agree that *Entamoeba williamsi* Prowazek 1911 is a mixture of *E. coli* and the amoeba of the "iodine cysts." Practically all observers agree that the species which Prowazek described the next year under the name *Entamoeba bütschlii* is entirely the amoeba of the "iodine cysts." The question arises, then, as to which name should be used for the amoeba of the "iodine cysts." If *williamsi* Prowazek, 1911, is to be considered a synonym of *E. coli*, *bütschlii* Prowazek, 1912, is the first available name. If, however, it can be shown that *williamsi* Prowazek, 1911, is available for the present form, it must take precedence over *bütschlii* Prowazek, 1912.

Castellani and Chalmers, 1913, and Hartmann, 1913, were the first authors to consider *E. williamsi* Prowazek. The former investigators considered it a probable synonym of *E. coli* but the latter clearly indicated that he considered it different from *E. coli* because he designated it *Entamoeba coli* var. *williamsi*. We have, therefore, two revisors of the same year for Prowazek's species and there is no way of fixing precedence by any dates given in the publications. We are, however, justified in accepting Hartmann's revision because he *definitely* stated that he considered *williamsi* different from *coli* whereas Castellani and Chalmers (1913, p. 269) gave *williamsi* as a *probable* synonym of *coli*. Another fact which necessitates our acceptance of Hartmann's revision is that the first subsequent investigator to recognize the nature of the organism under question (Brug 1919) and to designate the type material (Brug 1921) followed the spirit of Hartmann's revision. It is very important to note that Hartmann based his distinction between *coli* and *williamsi* on the motile amoeba and, therefore, if it can be shown that the motile stage of *williamsi* is different from *E. coli* we will have to retain the name *williamsi* for this new species, and it will not be permissible to consider it a synonym of *E. coli*. Not only have Nöller (1921) and Brug (1921) shown that Prowazek's original slides contained both *E. coli* and the amoeba of the "iodine cysts," but an examination of Prowazek's description of *E. williamsi* shows that the motile stages of this form (Figs. 1 and 2) are those of the "iodine cysts." A further fact of paramount importance is that Brug (1921) clearly designated Prowazek's original slides as the type material of the species. The revision of Prowazek's *williamsi* by Hartmann (1913), the designation of Prowazek's original material as the type material of *williamsi* by Brug (1921), and the identification of this material with the amoeba of the "iodine cysts" by Brug (1921) and Nöller (1921), make it quite clear to us that under the International Rules of Nomenclature (Articles 29, 30, and 31) *williamsi* is the valid name of the species in question.

To sum up, then, we feel that the name of the amoeba of the "iodine cysts" should be *Iodamoeba williamsi* Prowazek 1911.

Conclusions.

1. In the nucleus of the motile amoeba of the "iodine cysts" there is a well-defined nuclear membrane, a large central karyosome, a single layer of peripheral granules ("peripheral chromatin") which surrounds the karyosome, and a few fine linin strands which run from the karyosome through the layer of peripheral granules to the nuclear membrane. The peripheral granules show a different staining reaction from the karyosome.

2. The nucleus of the cysts contains the same structures as that of the motile amoebae. The karyosome, however, becomes eccentrically placed, and the peripheral granules, which vary greatly in number among different nuclei, become localized on one side of the karyosome, between it and the nuclear membrane.

3. With the exception of the linin strands, our description agrees with that of Dobell. The crescent-shaped mass which is described by Brug and Nöller in the nuclei of the cysts, is composed of the granules noted above. In cysts which are stained with iron haematoxylin and decolorized to too great an extent we have found many pictures which remind one very much of *E. nana*, and may explain the findings of Kofoid, Kornhauser and Swezy.

4. The structure of the nucleus of the amoeba of the "iodine cysts" makes it impossible to identify it with *Endolimax nana* or even to include it in the genus *Endolimax*.

5. The name of the amoeba of the "iodine cysts" should be *Iodamoeba williamsi* Prowazek 1911.

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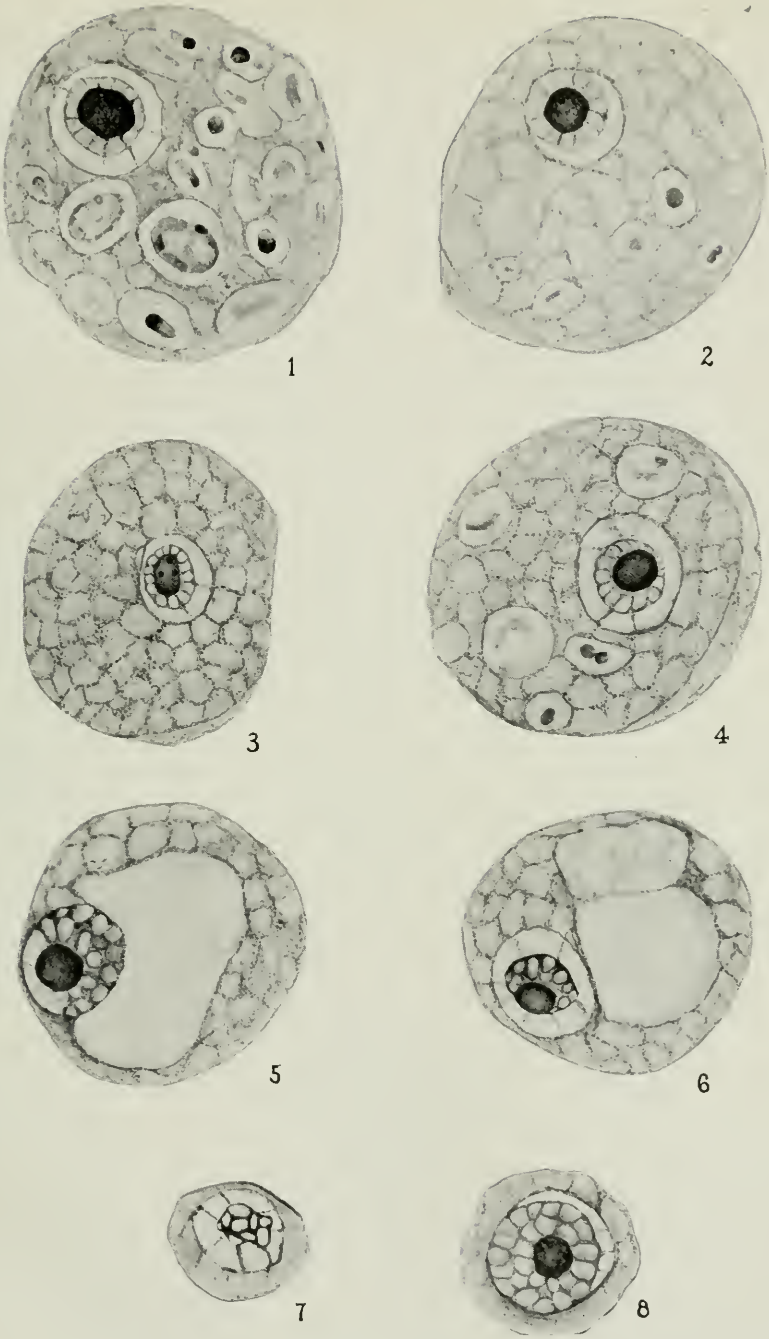
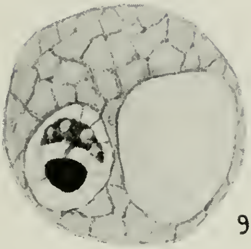


PLATE VIII.



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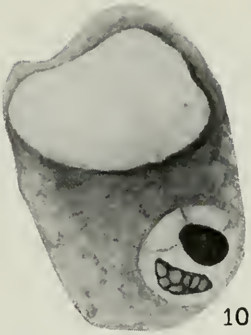

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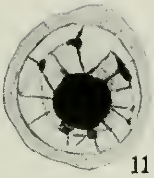

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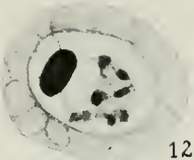

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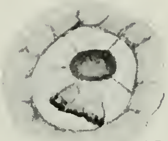

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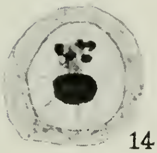

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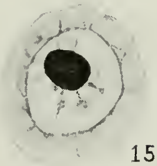

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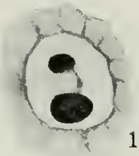

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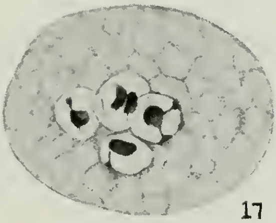

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PLATE IX.

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EXPLANATION OF PLATES.

All figures are camera lucida drawings of specimens fixed with sublimate-alcohol (with or without acetic acid), and stained as indicated. With the exception of Fig. 17, which is a cyst of *Endolimax nana*, all figures are drawings of *Iodamoeba williamsi.* Magnification × 4,300.

Plate VIII.

Figs. 1–2. Active amoebae stained with iron haematoxylin and eosin. The peripheral granules of the nucleus appear as a segmented ring surrounding the karyosome.

Fig. 3. Precystic amoeba stained with Mann's methylblue-eosin mixture. The peripheral granules surrounding the karyosome show very distinctly.

Fig. 4. Active amoeba stained as above. Note the linin strands running from the karyosome to the nuclear membrane.

Figs. 5–6. Cysts stained with Mann's methylblue-eosin mixture. Note the change in position of the karyosome and the peripheral granules. The glycogen masses have been dissolved, leaving the clear vacuoles shown in the cytoplasm.

Fig. 7. Nucleus of cyst, stained as above, but viewed from the pole toward which the peripheral granules are displaced.

Fig. 8. Nucleus of cyst, stained as above, but viewed from the pole toward which the karyosome is displaced.

Plate IX.

With the exception of Fig. 17, which is a cyst of *E. nana*, the drawings in this plate show incorrect nuclear pictures due to faulty staining. Magnification × 4,300.

Figs. 9–10. Cysts stained with iron-haematoxylin which show the presence of the "crescent-shaped mass" in the nucleus. This "crescent-shaped mass" is composed of the same peripheral granules as are shown in Figs. 5 and 6.

Figs. 11–16. Nuclei of cysts stained with iron-haematoxylin. These peculiar nuclear pictures are due to the way in which the mass of peripheral granules often behave in this stain. These figures are discussed fully in the text.

Fig. 17. Cyst of *Endolimax nana* stained with Mann's methylblue-eosin mixture. Compare with cysts shown in Figs. 5 and 6, and note the peculiar karyosome and absence of "peripheral chromatin" in this species.

THE RELATION OF BLOOD PLATELETS TO THE IN VIVO AGGLUTINATION OF BACTERIA AND THEIR DISAPPEARANCE FROM THE BLOOD STREAM.*

By CARROLL G. BULL, M.D., AND CLARA M. McKEE.

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PLATE X.

In the course of a study (1) of the mechanism by which the intravenous administration of immune serum causes an abrupt disappearance of the organisms from the blood stream of rabbits having a pneumococcic septicemia or bacteremia, it was observed that the pneumococci collected into clumps—were agglutinated—immediately after the serum was injected. It was further observed that the bacterial clumps rapidly left the circulation and collected in the capillaries of the internal organs—liver, spleen, lungs, etc. Similar observations (2) were made with other bacteria—dysentery bacilli, typhoid bacilli, staphylococci, etc. Virulent pneumococci were not agglutinated when injected into the circulation of normal rabbits. The conclusion was reached that there was a definite relation between the agglutination of bacteria in the blood stream and the resistance of the animal to septicemic infections, i.e., the greater the resistance of the animal, the more extensive the agglutination, and the more rapid the disappearance of the bacteria from the circulation. This was held to be true in the case of both natural and artificially acquired immunity, active or passive.

Since the publication of the above observations and conclusions other investigators have reported observations which tend to show that our deductions were too comprehensive, exceptions to the general conclusion being pointed out. Some have even questioned the occurrence of in vivo agglutination of bacteria. Thus Kitagawa (3) reports that an inagglutinable strain of pneumococci rapidly disappeared from the circulation of immune rabbits. Manwaring and Coe (4) found that pneumococci adhere to the endothelial cells of the hepatic capillaries of immune rabbits, while they are not retained by the liver of normal rabbits under the same conditions. The retention of the pneumococci by the endothelial cells is thought to result from the action of " endothelial opsonins " which, in turn, play an important rôle in the rapid disappearance of bacteria from the blood stream following artificial bacteremia. Tenbroeck (5) found that virulent hog-cholera bacilli were clumped and disappeared from the circulation immediately after being injected into the circulation of normal as well as of immunized rabbits. Hopkins and Parker (6) have reported that virulent streptococci first rapidly leave the circulation of susceptible animals and then return as the infection advances. These investigators concluded, however, that the streptococci were carried back into the circulation from infected tissues, and that they were dealing with a bacteremia instead of a true septicemia. This conclusion was reached because there was no evidence of the organisms having acquired properties which made permanent residence within the circulating blood possible. More recently Delrez and Govaerts (7) have published observations which seem to show that the blood platelets perform a general " antixenique " function, i.e., these elements are very active in the removal of all kinds of foreign matter from the circulation, and possibly also in the destruction of bacteria by the process of phagocytosis and intracellular digestion. They also conclude that a genuine agglutination of bacteria does not occur in the circulation of normal animals—the phenomenon observed by one of us not being true agglutination. Meyer, Neilson and Fensier (9) are inclined to believe that in vivo agglutination does not occur, and state that the disappearance of bacteria from the blood stream is probably not due to agglutination, but to the action of the blood platelets or to a dispersion phenomenon of two colloids.

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* From the Department of Immunology, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.

We are not prepared at present to offer an opinion on all the reports which we have mentioned, since we have not had the opportunity of studying them This paper concerns the one point which we have studied, namely: the relation of the blood platelets to the agglutination of bacteria in the blood stream of animals having an artificial bacteremia, and the part which these elements of the blood play in the abrupt disappearance of bacteria from the circulation.

The platelet theory has as its basis the fact that when certain bacterial suspensions are injected into the circulation of rabbits (or other animals) the injected bacteria and the platelets collect, within a few seconds time, into more or less compact clumps (Fig. 1, Plate

X) and that the clumps soon leave the circulating blood. This phenomenon can easily be observed by injecting a suspension of Flexner dysentery bacilli into the ear vein of a normal rabbit, or a suspension of pneumococci into the ear vein of an immune rabbit, and taking specimens of blood from the heart from 30 seconds to 2 minutes after the injection is made. The part of the theory which concerns the destruction of the bacteria by the platelets is purely hypothetical.

This association of the platelets with the clumps of bacteria, or of the bacteria with the clumps of platelets, was observed in our first work on the mechanism of the abrupt disappearance of the bacteria from the circulation of resistant animals, but it was not considered to be essential to the phenomenon of in vivo agglutination, because platelets were not always attached to or mixed with the bacterial clumps (Fig. 2, Plate X), particularly in the case of immunized animals. We have attempted to answer three questions, namely: (1) Are platelets essential to the collection into masses (agglutination) of bacteria in immunized animals? (2) in normal animals? and (3) do the platelets play an essential rôle in the abrupt disappearance of bacteria from the circulation of animals in which an artificial bacteremia is produced? The general plan of study was to make identical tests in normal rabbits and in rabbits whose blood platelets had been destroyed or removed from the circulation.

EXPERIMENTAL.

General technique.—The technique of the experiments was in part the same as that previously described (1). From 2 to 5 c.c. of bacterial suspensions, of suitable densities, were injected into the ear veins of rabbits, and specimens of blood for microscopic observation and culturing were taken from the heart at intervals varying from 5 seconds to 2 hours after the injections were made. When passively immunized animals were being tested the immune serum was given intravenously a few minutes before the bacteria were injected. Platelet counts were made by Wright's method, which proved to be satisfactory for our purpose. The animals were " deplateletized " by the intravenous administration of an anti-platelet serum which was prepared by giving guinea pigs three or four intraperitoneal injections of the washed platelets from 50 to 60 c.c. of rabbit blood. The guinea pigs were bled from 7 to 10 days after the last injection, and in every instance a serum was obtained which caused the platelets to disappear rapidly from the circulation of rabbits without affecting the leucocytes or erythrocytes.

IMMUNIZED ANIMALS.

Active acquired immunity.—The first series of experiments deals with actively immunized rabbits. A number of rabbits were immunized with each of the following organisms: (*a*) pneumococci, (*b*) Flexner dysentery bacilli, (*c*) typhoid bacilli, and (*d*) *Staphylococcus aureus.* The details of the individual tests are given in the following representative protocols.

Experiment 1 A: A rabbit, weight 2,150 grams, had been given four intravenous injections of killed Type 1 pneumococci. Its serum agglutinated the homologous organisms in a dilution of 1–25. A platelet count gave 375,000 per c.mm. Three c.c. of antiplatelet serum were injected into the ear vein. Two hours later the platelets had fallen to 6,000* per c.mm., and no platelets could be found in a stained specimen of blood. 80,000 million pneumococci were injected into the ear vein, and specimens of blood were taken from the heart for culture and microscopic examination as indicated in Protocol A.

PROTOCOL A.

Agglutination of pneumococci in a " deplateletized " immune rabbit.

Time after pneumococci were injected.	Specimen of heart blood.	Colonies per c.c.	Bacterial clumps.	Platelets.
0′ 5″*........	1st	7,860,000	many	none
0′ 40″........	2d	2,000,000	fewer	"
1′ 5″........	3d	1,200,000	"	"
3′ 15″........	4th	65,000	none	"
5′ 55″........	5th	20,000	"	"
10′ 30″.......	6th	5,000	"	"

Experiment 1 B: The rabbit used in this test weighed 2,000 grams, and had been immunized with killed cultures of Type 1 pneumococci; its serum agglutinated the homologous organisms in a dilution of 1–50. A platelet count gave 350,000 per c.mm. of blood. 80,000

million pneumococci were injected into the ear vein and specimens of blood were taken from the heart for culture and microscopic examination as shown in Protocol B.

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* Since it is not possible to distinguish with certainty a blood platelet from particles of other materials in the counting chamber, such low platelet counts mean that there were not more and probably fewer platelets present than the figures indicate.

* ′—minutes, ″—seconds.

PROTOCOL B.

Agglutination of pneumococci in a "normal" immune rabbit.

Time after pneumococci were injected.	Specimen of heart blood.	Colonies per c.c.	Bacterial clumps.	Platelets.
0′ 5″........	1st	7,200,000	many	numerous
0′ 45″........	2d	1,850,000	fewer	"
1′ 10″........	3d	1,020,000	"	"
3′ 30″........	4th	70,000	none	"
5′ 50″......	5th	15,000	"	"
10′ 30″........	6th	6,000	"	"

Protocols A and B show that pneumococci agglutinate in the circulation of an actively immunized rabbit whose platelets have been destroyed, or removed from the blood stream, just as quickly and completely as they do in a rabbit having the normal number of platelets.

It is also seen that the cocci leave the circulation with equal rapidity in the two rabbits, the microscopic examinations being negative in each case after 3½ minutes. The three first specimens of blood from the rabbit (Protocol B) whose platelets had not been destroyed showed many clumps (Fig. 3, Plate X) of the diplococci, entirely free of platelets, and also many clumps with platelets mixed with them. There were numerous clumps of platelets free of cocci. The third specimen had the greatest proportion of mixed clumps. One concludes, therefore, that under the conditions of these experiments, the association of the platelets with the bacterial clumps is merely incidental, since the agglutination and disappearance of the bacteria from the circulation are not perceptibly modified by their presence.

Experiments similar to 1 A and 1 B were made with rabbits which had been immunized with *B. typhosus*, *B. dysenteriae* Flexner, and *Staphylococcus aureus*. The results were the same as those obtained with pneumococci (Figs. 4, 5 and 6, Plate X), with the exception that there were, in the rabbits whose platelets had not been destroyed, fewer clumps of bacteria with which no platelets were mixed. The bacteria left the blood stream of the deplateletized rabbits as rapidly as that of the normal rabbits.

Passive acquired immunity.—The experiments just described were repeated in rabbits passively immunized to the different bacteria. The bacteria were agglutinated and disappeared from the circulation of the rabbits whose platelets had been destroyed just as quickly and completely as they did in the controls. Experiments 2 A and 2 B with typhoid bacilli will serve to illustrate the results obtained with the passively immunized animals.

Experiment 2 A: A rabbit weighing 1,800 grams was given 3 c.c. of antiplatelet serum. Two hours later a platelet count gave 7,000 per c.mm., and no platelets could be found in stained blood films. 10 c.c. of antityphoid serum was injected into the ear vein and two minutes later 70,000 million typhoid bacilli were injected into the other ear vein. Specimens of blood were taken from the heart for culture and films, as indicated in Protocol C.

PROTOCOL C.

Agglutination of typhoid bacilli in a passively immune "deplateletized" rabbit.

Time after bacilli were injected.	Specimen of heart blood.	Colonies per c.c.	Bacterial clumps.	Platelets.
0′ 5″.........	1st	20,640,000	many	none
0′ 55″.........	2d	6,500,000	fewer	"
2′ 15″.........	3d	2,006,000	"	"
4′ 15″.........	4th	500,000	"	"
9′ 5″....... .	5th	60,000	none	"

Experiment 2 B: A rabbit, weight 1,850 grams, platelet count 450,000 per c.mm., received 10 c.c. of antityphoid serum in the vein and one minute later 70,000 million typhoid bacilli. Protocol D gives the details of the cultures and films of the blood specimens.

Throughout the experiments on immunized animals it was noticed that the first specimen taken after the bacteria were injected contained the greatest number of bacterial clumps without platelets. In some instances, particularly with pneumococci and dysentery bacilli, a great majority of the clumps had no platelets associated with them. The subsequent specimens contained more and more clumps of bacteria mixed with platelets. This observation leads one to believe that the bacteria are agglutinated before the platelets become attached to them. This point will be referred to again in connection with the experiments on normal rabbits.

PROTOCOL D.

Agglutination of typhoid bacilli in a passively immune " normal " rabbit.

Time after bacilli were injected.	Specimen of heart blood.	Colonies per c.c.	Bacterial clumps.	Platelets.
0′ 5″.........	1st	18,500,000	many*	numerous
0′ 30″.........	2d	3,500,000	fewer	"
1′ 50″.........	3d		"	"
3′ 50″.........	4th	2,800,000	"	"
6′ 0″.........	5th	750,000	"	"
9′ 50″.........	6th	100,000	"	"

NORMAL ANIMALS.

A series of experiments was made with normal rabbits. The general technique was similar to that just described in connection with the experiments on immunized rabbits. Two rabbits of equal size were used in each test, one being deplateletized and the other not. In some instances, particularly in the experiments with *B. typhosus,* the titer of the natural agglutinins was determined and rabbits having equal titers were chosen for the individual tests. Three species of bacteria were used, namely: *B. typhosus, B. dysenteriae* (Flexner), and *Staphylococcus pyogenes aureus.* The details of the experiments are given in the following type protocols.

B. dysenteriae (Flexner).—A large number of tests were made with this organism. Since the results were regularly similar, one detailed experiment (Experiment 3) will suffice for the purpose of illustration.

Experiment 3: A platelet count was made on each of three rabbits. The number of platelets per c.mm. were, respectively, 245,000, 260,000, and 395,000. The rabbit (Protocol G) which had the highest platelet count was given 2 c.c. of antiplatelet serum in the ear vein, and 45 minutes later the count was 7,000. The rabbits were now tested for in vivo agglutination and the rate of disappearance of the bacilli from the blood stream. Each rabbit received an intravenous injection of 75,000 million dysentery bacilli, and blood specimens were taken from the heart as indicated in the respective protocols.

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* In the first specimen of blood there were many clumps of bacilli which were free of platelets, in the second specimen only a few and in the other specimens all of the clumps were attached to clumps of platelets.

Protocol E.

Agglutination of dysentery bacilli in a normal rabbit.

Time after bacilli were injected.	Specimen of heart blood.	Clumps of bacilli.	Clumps of bacilli and platelets.	Free bacilli.
0′ 5″.........	1st	+	0	+++
0′ 30″.........	2d	+++	0	+
0′ 50″.........	3d	++	++	+
0′ 10″.........	4th	0	+	+
1′ 30″.........	5th	0	+	+ −
1′ 50″.........	6th	0	+	0

Protocol F.

Agglutination of dysentery bacilli in a normal rabbit.

Time after bacilli were injected.	Specimen of heart blood.	Clumps of bacilli.	Clumps of bacilli and platelets.	Free bacilli.
0′ 10″.........	1st	+	0	++
0′ 50″.........	2d	++	+	+
1′ 30″.........	3d	0	++	+
2′ 5″.........	4th	0	+	+ −
2′ 40″.........	5th	0	+	0
3′ 30″.........	6th	0	+	0

Protocol G.

Agglutination of dysentery bacilli in a normal " deplateletized " rabbit.

Time after bacilli were injected.	Specimen of heart blood.	Clumps of bacilli.	Clumps of bacilli and platelets.	Free bacilli.
0′ 10″.........	1st	++	0	+
0′ 38″.........	2d	+++	0	+
1′ 12″.........	3d	+	0	+
1′ 54″.........	4th	+	0	+
2′ 36″.........	5th	+	0	0
3′ 39″.........	6th	0	0	0

Protocols E, F, and G present two points of interest. First, the absence of the platelets did not perceptibly influence the agglutination of the bacilli (Fig. 7, Plate X) or their disappearance from the blood stream. Second, the first and second specimens of blood from one of the rabbits (Protocol E) whose platelets had not been removed and the first specimen from the other (Protocol B) contained only clumps of bacilli with which no platelets were mixed. The following specimen in each case contained clumps of bacilli, both with and without an admixture of platelets, while in the later specimens there were only mixed clumps. This observation indicates that here again the bacteria are first agglutinated and then come in contact with clumps of platelets.

B. typhosus.—Since the sera of normal rabbits show considerable variations with respect to agglutination of typhoid bacilli, the serum of each rabbit was titrated in vitro, before the in vivo tests were made. The sera were heated at 56° C. for thirty minutes and the titers determined by the macroscopic method. The titers varied from a 1–2 to a 1–16 dilution of serum. The individual tests were made on rabbits having equal agglutinating titers. Experiment 4 illustrates the results.

Experiment 4: Three rabbits were used in this experiment. Two had agglutinating titers of 1–4, the other 1–16. One of the rabbits with a 1–4 agglutinating titer (Protocol I) and the one with the 1–16 titer (Protocol J) were given antiplatelet serum. The other rabbit (Protocol H) was used as a control. Each rabbit was given an intravenous injection of 75,000 million typhoid bacilli, and specimens of blood were taken from the heart at short intervals and examined microscopically to determine the extent of the agglutination of the bacilli and the rate of their disappearance from the circulation. The observations are recorded in Protocols H, I, and J.

PROTOCOL H.

Agglutination of typhoid bacilli in a normal rabbit (control).

Time after bacilli were injected.	Specimen of heart blood.	Clumps of bacilli.	Clumps of bacilli and platelets.	Free bacilli.
0′ 7″........	1st	0	0	+++
0′ 47″........	2d	+	+	++
2′ 21″........	3d	0	++	+
4′ 18″........	4th	0	+++	+
7′ 22″........	5th	0	++	+
10′ 1″........	6th	0	+	+
17′ 13″........	7th	0	0	+
20′ 3″........	8th	0	0	+

PROTOCOL I.

Agglutination of typhoid bacilli in a normal "deplateletized" rabbit.

Time after bacilli were injected.	Specimen of heart blood.	Clumps of bacilli.	Clumps of bacilli and platelets.	Free bacilli.
0′ 11″........	1st	++	0	++
1′ 0″........	2d	+++	0	+
2′ 4″........	3d	++	0	+
4′ 0″.......	4th	+	0	+
7′ 30″........	5th	+	0	+
10′ 15″........	6th	0	0	+
15′ 21″........	7th	0	0	+
20′ 19″........	8th	0	0	0

PROTOCOL J.

Agglutination of typhoid bacilli in a normal "deplateletized" rabbit.

Time after bacilli were injected.	Specimen of heart blood.	Clumps of bacilli.	Clumps of bacilli and platelets.	Free bacilli.
0′ 7″.........	1st	++	0	++
0′ 23″.........	2d	+++	0	+
1′ 5″.........	3d	++	0	+
1′ 35″.........	4th	++	0	+
2′ 30″.........	5th	+	0	+
3′ 31″.........	6th	+	0	+
4′ 10″.........	7th	0	0	0
5′ 2″.........	8th	0	0	0

It is shown by Experiment 4 that typhoid bacilli agglutinate (Fig. 8, Plate X) in the blood stream of normal rabbits, and also that the bacilli rapidly leave the circulation. Both the agglutination and the disappearance of the bacilli from the circulation seem to be directly related to the potency of the natural agglutinins rather than to the presence of the blood platelets. The agglutinating titers of the sera of the rabbits used in Protocols H and I were the same, 1–4. The bacilli left the circulation with about equal rapidity. The difference was in favor of the deplateletized rabbit (Protocol I), no bacilli being found in the films of the 20-minute specimen of blood, while they were found in the corresponding specimen from the control rabbit. Agglutination occurred in both instances. In the normal rabbit (Protocol H) platelets were mixed with all of the clumps, with the exception of the second specimen, which contained some platelet-free clumps of bacilli, while there were no platelets in any of the specimens from the deplateletized rabbit. In both cases the unagglutinated bacilli were the last to disappear. In Protocol J no bacilli were found in the specimens taken 4 minutes and 10 seconds after the bacteria were injected. This rabbit had been deplateletized, but the serum had a high agglutinating titer, 1–16.

Other similar tests were made with rabbits whose sera had very low agglutinating titers. The bacilli were only slightly agglutinated and remained in the circulation a comparatively long time, no appreciable reduction in the number of organisms occurring within ten minutes after the injections were made. The results were the same, however, in the normal and the deplateletized rabbits.

Staphylococcus pyogenes aureus.—This organism was selected because the sera of normal rabbits agglutinate it only slightly, or not at all. A large number of rabbits were tested for natural agglutinins, and none of the sera agglutinated the cocci within two hours at 37° C. If the tests were kept at 37° C. for two hours and then allowed to remain at room temperature for 18 hours, agglutination occurred in some instances in 1–4 and 1–8 dilutions of serum. However, when a rabbit was tested several times, at short intervals, the results were very irregular, and the presence or absence of natural agglutinins was disregarded. The procedure was the same as with the other bacteria, i.e., the rabbits were paired, one being given antiplatelet serum and the other not. The blood specimens were both plated and examined microscopically. A number of tests were made, from 14,000 million to 80,000 million cocci being injected, but since the results were uniformly similar only two experiments will be given in detail.

Experiment 5 A: Two rabbits of equal weight were selected. One had a platelet count of 375,000, the other 400,000. The latter (Pro-

PROTOCOL K.

Disappearance of staphylococci from the blood stream of a normal rabbit.

Time after cocci were injected.	Specimen of heart blood.	Colonies per c.c.	Clumps of platelets and cocci.	Free cocci.
0′ 15″........	1st	innumerable (0.005 cc.)	few cocci on clumps of platelets	many
15′ 15″........	2d	31,000	no cocci	none
36′ 0″........	3d	15,000	" "	"
56′ 0″........	4th	10,000	" "	"

tocol L) was given 3 c.c. of antiplatelet serum. Both rabbits were given 15,000 million staphylococci in the ear vein, and four specimens of blood were taken from the heart, at intervals of about 15 minutes. See Protocols K and L.

PROTOCOL L.

Disappearance of staphylococci from the blood stream of a " deplateletized " normal rabbit.

Time after cocci were injected.	Specimen of heart blood.	Colonies per c.c.	Clumps of platelets and cocci.	Free cocci.
0′ 10″........	1st	innumerable (0.005 cc.)	no platelets	many
15′ 15″........	2d	30,000	" "	none
31′ 5″........	3d	11,000	" "	"
65′ 35″........	4th	2,400	" "	"

PROTOCOL M.

Disappearance of staphylococci from the blood stream of a normal rabbit.

Time after cocci were injected.	Specimen of heart blood.	Colonies per c.c.	Clumps of platelets and cocci.	Free cocci.
0′ 10″.........	1st	innumerable (0.005 cc.)	few cocci on clumps of platelets	many
0′ 34″.........	2d	innumerable	many cocci on clumps of platelets	"
0′ 48″.........	3d	15,860,000	many cocci on clumps of platelets	fewer
2′ 13″.........	4th	1,060,000	very few on clumps of platelets	"
2′ 57″.........	5th	950,000	very few on clumps of platelets	"
3′ 51″.........	6th	600,000	very few on clumps of platelets	"
5′ 10″.........	7th	540,000	very few on clumps of platelets	"
7′ 0″.........	8th	350,000	very few on clumps of platelets	"

Experiment 5 B: Two rabbits, each weighing 2,000 grams, had platelet counts of 425,000 and 372,000 respectively. The rabbit having the lowest platelet count was given 3 c.c. of antiplatelet serum. Both rabbits were given 14,000 million staphylococci in the ear vein and eight specimens of blood were taken from the heart during the succeeding 7 minutes. The details are given in Protocols M and N.

PROTOCOL N.

Disappearance of staphylococci from the blood stream of a "deplateletized" normal rabbit.

Time after cocci were injected.	Specimen of heart blood.	Colonies per c.c.	Clumps of platelets and cocci.	Free cocci.
0′ 11″.........	1st	innumerable (0.005 cc.)	no platelets	many
0′ 31″.........	2d	innumerable	" "	"
0′ 49″.........	3d	7,460,000	" "	"
1′ 38″.........	4th	2,300,000	" "	fewer
2′ 25″.........	5th	550,000	" "	"
3′ 30″.........	6th	140,000	" "	"
5′ 17″.........	7th	90,000	" "	"
6′ 3″.........	8th	40,000	" "	none

Experiments 5 *A* and 5 *B* show that the blood platelets do not play an essential rôle in the disappearance of staphylococci from the blood stream of normal rabbits, and also that in vivo agglutination does not occur, except possibly to a slight degree in some instances. In the first experiment (5 *A*) the blood specimens were taken from 10 seconds to about one hour after the cocci were injected into the ear vein, and it is seen that comparatively few organisms are in the blood at the expiration of 15 minutes, and that the number is only slightly reduced after this time. There was no appreciable difference in the rate of disappearance in the normal and the deplateletized rabbit, the slight difference being in favor of the rabbit whose platelets had been destroyed. Experiment 5 *B* covers only the first phase of the disappearance curve. Here again cocci left the circulation of the deplateletized rabbit somewhat more rapidly than that of the normal rabbit. Our conclusion is that the platelets do not materially influence the disappearance curve, the discrepancies in the number of organisms in the corresponding blood specimens being due to variations in other respects in the individual rabbits and to imperfections of the tech-

nique. At least, the absence of the platelets does not retard the disappearance of the bacteria from the circulating blood. The collection of the cocci on clumps of platelets must be, therefore, an incidental phenomenon.

Discussion.

The observations recorded above show quite definitely that bacteria are agglutinated immediately after being injected into the blood stream of immunized animals. The reaction is completed within a few minutes, and the clumped bacteria disappear from the circulating blood. In normal rabbits, i.e., rabbits whose platelets have not been destroyed, platelets are frequently mixed with the bacterial clumps. It is evident, however, that the platelets are not essential to the formation of the bacterial clumps since, even with the normal number of platelets present, many clumps have no platelets associated with them. It is indicated, moreover, that in the formation of the mixed clumps (platelets and bacteria) the bacteria are first agglutinated and then pick up the platelets, because the earlier specimens of blood contain many more platelet-free clumps of bacteria than the later specimens. The experiments with immunized rabbits whose blood was practically free of platelets show conclusively that the blood platelets are in no way essential to the phenomenon of in vivo agglutination, and the abrupt disappearance of the bacteria from the blood stream. With normal rabbits the results are variable and depend upon the kind of bacteria used. Flexner dysentery bacilli regularly behave in normal rabbits as pneumococci do in immunized rabbits, i.e., they are actively agglutinated and leave the circulation independently of any influence of the platelets. Varying results were obtained with typhoid bacilli, particularly with regard to agglutination. If the serum contained few or no agglutinins for this organism, there was little or no clumping of the bacilli in deplateletized rabbits, but they collected on clumps of platelets in the control rabbits. If the serum contained more agglutinins, the bacilli were agglutinated and rapidly left the blood stream in the absence of platelets. The clumping and disappearance of the bacilli from the circulation were directly related to the agglutinin content of the serum, and were independent of the number of platelets present. *Staphylococcus aureus* did not agglutinate in the normal rabbits whose platelets had been destroyed, but collected, to a greater or less extent, on clumps of platelets in rabbits having the normal number of platelets. But even in this case the cocci left the circulating blood just as quickly in the deplateletized as in the control rabbits. It is concluded, therefore, that neither the agglutination of bacteria within the blood stream nor their disappearance from it can be ascribed to a function of the platelets.

It now seems possible to define clearly the sequence of events which occurs when bacterial suspensions are injected into the blood stream. If the animal possesses an appreciable amount of agglutinins the bacteria are immediately agglutinated and rapidly leave the blood stream, collecting in the capillary systems of the liver, spleen, lungs, etc. As the clumps of bacteria form they may become associated with clumps of platelets, before leaving the blood stream, but the platelets are not essential either to the agglutination or to the disappearance of the bacteria from the blood stream, since they both occur when the blood contains no platelets. If the bacteria are not agglutinated, but are so affected as to make them more " adhesive " (opsonized?) they become attached to clumps of platelets and then collect in the capillary systems. Here, again, the platelet phase is merely incidental, for when platelets are not present the bacteria adhere directly to the capillary endothelium, leaving the circulating blood as quickly as when the platelets are present. If the bacteria are neither agglutinated nor opsonized, as is the case with virulent pneumococci in normal rabbits, they do not leave the blood stream abruptly, but continue to circulate in the blood and produce a septicemia without a reduction in the number of organisms having occurred. The " adhesive " effect mentioned above is considered to be a preagglutination effect, i.e., if the bacteria were more intensely affected agglutination would result. It is due to such an action on the part of the plasma that staphylococci rapidly leave the blood stream of normal rabbits and collect in the viscera, and it offers an explanation of the observations of Kitagawa (3) on inagglutinable pneumococci and of Manwaring and Coe (4) on the collection of pneumococci in the hepatic capillaries, as the result of treating the organisms with an immune serum which was diluted beyond its agglutinin titer. The plasma of normal rabbits does not affect virulent pneumococci even to this extent, and consequently these organisms remain in the blood stream.

SUMMARY AND CONCLUSIONS.

1. When bacterial suspensions are injected into the circulation of immunized rabbits, or of normal rabbits having natural agglutinins,

the bacteria are immediately agglutinated and rapidly leave the blood stream. The blood platelets, when present, become attached to a majority of the clumps of bacteria, but both the agglutination and the disappearance of the bacteria from the circulation, of either normal or immunized animals, are just as complete and rapid, when the platelets are absent.

2. Suspensions of *Staphylococcus aureus* are not agglutinated, when injected into the circulation of normal rabbits, but the bulk of the organisms rapidly leave the blood stream. When platelets are present they collect into clumps to which many of the cocci become attached before leaving the circulation, but the cocci leave the circulation with equal rapidity when no platelets are present. In this case the bacteria leave the blood stream because the plasma so alters them that they adhere to the endothelial cells of the capillary systems.

3. Virulent pneumococci remain in the circulation of normal rabbits, because they are neither agglutinated nor opsonized.

4. Depending upon the antibody content of the blood, one of three things occurs when artificial bacteremias are produced, viz.: (1) the bacteria are neither agglutinated nor opsonized, and continue to circulate in the blood stream, (2) the bacteria are agglutinated, or are both agglutinated and opsonized, and rapidly leave the circulating blood and collect in the capillary systems of the liver, spleen, lungs, etc., or (3) the bacteria are not agglutinated, but are opsonized and rapidly leave the circulating blood and likewise collect in the internal organs.

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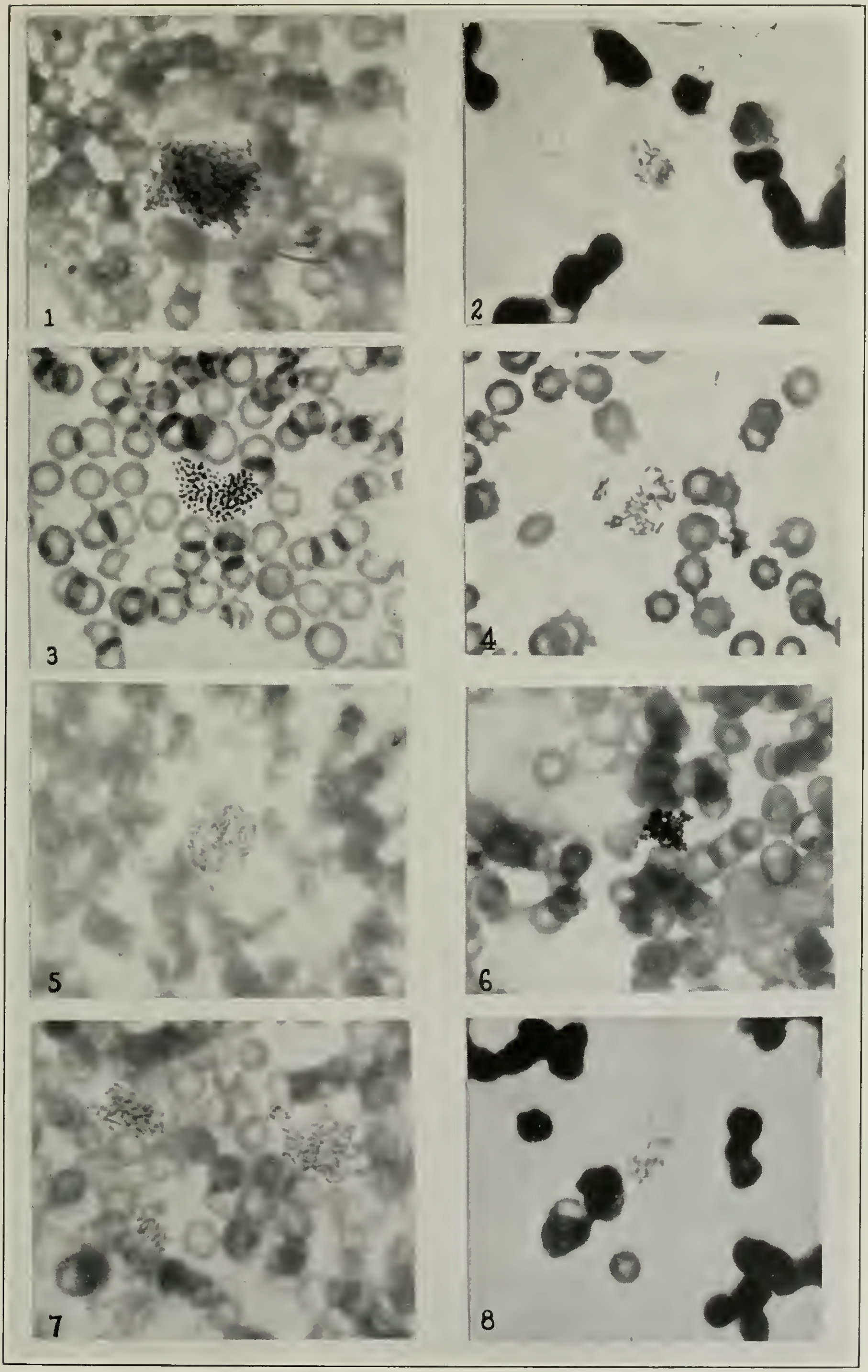

PLATE X.

PROCEEDINGS OF THE SOCIETY OF HYGIENE OF THE SCHOOL OF HYGIENE AND PUBLIC HEALTH OF THE JOHNS HOPKINS UNIVERSITY, BALTIMORE, MD.

Regular Meeting of Monday, February 13, 1922.

The Cost of the Reproduction Effort, Measured from the Date of Conception; Based on Maryland Birth Registration, and Applying Pearson's Formula for Prenatal Time. By John S. Fulton, M.D., Maryland State Board of Health.

Birth Registration in Maryland 1919 and 1920 showed that 69,813 women bore 76,213 infants; 6,400 more infants than mothers. Plural births accounted for 1,543 children of 768 mothers. The excess of children over mothers is thus reduced to 4,857.

It is possible, but not likely, that 4,857 mothers can have been parturient twice in a year. The smallest number of women able to account for 4,857 children is 2,375 mothers, of whom 53 would have had twins twice and 2,322 women would have had singles twice in a year. The true explanation is between these limits and is the frequency of women twice parturient in twelve months.

The Pearsonian factor for prenatal time (nine months) is 605 children dying in utero for each one thousand born alive at term. Of these prenatal deaths, 391 occur in the earliest three months, 131 in the second, and 83 in the last three months.

Therefore starting with 70,453 children registered born alive in Maryland we must add 42,624 children conceived and lost in utero. There were 113,077 children conceived. Lost in the first three months were 27,547; in the second three months 9,229; in the last three months 5,848.

The prenatal mortality accounted for by registration is about equivalent to the theoretical number for the last third of pregnancy. 5,760 stillbirths were recorded, 88 fewer than the theoretical loss in the last three months.

Nineteen thousand primiparae gave ages ranging from eleven to fifty-six years. The Modal age was 20.7 years, the Median 22.03

years, Mean 22.7 years. Parturition was fatal to 536 women, a fatality rate of 7.7 per 1000.

The Resistance of Different Hosts to Trypanosome Infections. By W. H. Taliaferro and L. G. Taliaferro. (From the Department of Medical Zoölogy of the School of Hygiene and Public Health of the Johns Hopkins University.)

In the present work we have used the term resistance to include any active or passive condition arising in the host which affects the trypanosomes adversely. Any resistance may do one or both of two things: (1) it may retard the rate of reproduction of the parasites, or (2) it may destroy them after they are formed. A fairly accurate picture of the sum total of the resistance of the host may be obtained by making daily determinations of the number of trypanosomes per cc. of blood. If, for example, the parasites either remain constant or decrease in number for a certain period, we know some type of resistance is operative, but we do not know whether it is producing its effect by retarding the rate of production of the parasites or by destroying them after they are formed. In the present work we have supplemented such an enumerative study with a measure of the rate of reproduction of the trypanosomes. This differentiates between the two types of resistance because of the following equation:

$$\begin{array}{l}\text{Number of parasites}\\ \text{per cc. of blood}\end{array} = \begin{array}{l}\text{Number produced by}\\ \text{reproduction of the}\\ \text{parasites}\end{array} - \begin{array}{l}\text{Number destroyed in}\\ \text{consequence of the}\\ \text{resistance}\end{array}$$

Although our measure of the rate of reproduction of the parasites is a comparative one, it is independent of the number destroyed in consequence of the resistance. It is based on the fact that the coefficient of variation for factors involving size (such as total length) is higher in a sample drawn from a population in which rapid reproduction is taking place than for a sample from a population in which there is little or no reproduction. This follows because the first consists of organisms in all stages of growth, whereas the second consists mainly of adult forms which show little variation in size. Furthermore, the greater the rate of reproduction with its production of young forms the greater the coefficient of variation, and, as the increase in the coefficient of variation is more or less proportional to the increase in the rate of reproduction, it can be used as a measure of the latter.

Studying resistance along these lines, we have found three conditions of affairs: (1) In non-pathogenic infections, such as *T. lewisi* in the rat, a resistance develops which both retards the rate of reproduction of the parasites and destroys them after they are formed. (2) In relapsing infections with pathogenic species, such as *T. brucei* and *rhodesiense* in the guinea pig and dog, a resistance develops which destroys large numbers of the parasites, but does not affect the rate of reproduction of the remaining organisms. (3) In continuous infections with pathogenic species, such as *T. equiperdum* and *rhodesiense* in the rat and mouse, very little, if any, resistance develops, either retarding the rate of reproduction or destroying them after they are formed.

(This paper will appear in full in the May issue of the JOURNAL.)

Correlation between any two Functions of a Set of Variables. By LOWELL J. REED. (From the Department of Biometry and Vital Statistics of the School of Hygiene and Public Health of the Johns Hopkins University.)

One of the earliest applications of the correlation coefficient occurred in the problem of finding the relationships existing between the lengths of different bones of the body. In this problem it was found necessary to deal with the relative lengths of the bones rather than with their absolute values. The calculation of the coefficient of correlation of these ratios of bone length was the first case of the application of the method of correlation to functions of variables rather than to the variables themselves. For the purpose of finding the correlation in this case, Pearson* developed a formula for the correlation between two indices. Since this early use of correlation statisticians have found it necessary to correlate functions of the variables that are more complex than simple ratios. All of the statistical index numbers now in use are functions of certain variables. For example the index number $(b-d)/p$, which has been used as an index of the virility of peoples, is a function of the three variables, b the number of births, d the number of deaths and p the size of the population. The increase in the number of functions to which the method of correlation is applied has made desirable a general formula by means of which we may determine the correlation between any two functions. For this purpose the following formula has been developed.

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* Pearson, K. On a Form of Spurious Correlation which may arise when Indices are used in the Measurement of Organs. *Proc. Roy. Soc. of London*, Vol. 60.

Let $x_1, x_2, \cdots, x_n$ be a set of variables and $x_1', x_2', \cdots, x_k'$ be a second set of variables.

Let $m_1, m_2, \cdots$, etc. and $\sigma_1, \sigma_2, \cdots$, etc. be the means and standard deviations of the variables. Then if $y = f(x_1, x_2, \cdots, x_n)$ and $Z = F(x_1', x_2', \cdots, x_k')$ are any two functions of these sets of variables, we have for the correlation between these two functions

$$r_{yz} = \frac{\sum_{\alpha=1}^{n}\sum_{\beta=1}^{k} f_\alpha F_\beta r_{x_\alpha x'_\beta}\sigma_\alpha\sigma_\beta'}{\sqrt{\left[\sum_{\alpha=1}^{n}\sum_{\beta=1}^{n} f_\alpha f_\beta r_{x_\alpha x_\beta}\sigma_\alpha\sigma_\beta\right]\left[\sum_{\alpha=1}^{k}\sum_{\beta=1}^{k} F_\alpha F_\beta r_{x'_\alpha x'_\beta}\sigma_\alpha'\sigma_\beta'\right]}} \tag{1}$$

where $r_{x_\alpha x'_\beta}$ denotes the correlation between the variables x_α, x_β' and

$$f_\alpha = \frac{\partial f(m_1, m_2, \cdots, m_n)}{\partial m_\alpha}$$

$$F_\beta = \frac{\partial F(m_1', m_2', \cdots, m_k')}{\partial m_\beta'}.$$

Equation (1) thus furnishes the coefficient of correlation of the two functions f and F in terms of the means, standard deviations and correlation coefficients of the variables themselves. The formula should not be applied to cases where the standard deviation is large with respect to the mean since terms of the second and higher order in $\sigma_1/m_1, \sigma_2/m_2, \cdots$ have been dropped from the equation.

One of the most important uses for formula (1) is in the case of spurious correlation. For a discussion of spurious correlation in the case of two ratios the reader should consult the previously mentioned article by Pearson. In the general case, spurious correlation may be said to arise whenever the two functions f and F have certain variables in common. If we suppose that the variables $x_1, x_2, \cdots, x_h$ are common to our two sets of variables and represent the amount of spurious correlation by ρ_{yz}, we have

$$\rho_{yz} = \frac{\sum_{\alpha=1}^{h} f_\alpha F_\alpha \sigma_\alpha^2}{\sqrt{\left[\sum_{\alpha=1}^{n} f_\alpha^2\sigma_\alpha^2\right]\left[\sum_{\alpha=1}^{k} F_\alpha^2\sigma_\alpha'^2\right]}} \tag{2}$$

Whenever two functions have been correlated and these functions contain common variables the amount of spurious correlation as given by equation (2) should be taken into consideration.

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A NOTE ON THE INHERITANCE OF DURATION OF LIFE IN MAN.*

BY RAYMOND PEARL.

(Received for publication January 2d, 1922.)

Experimental studies on *Drosophila* have definitely demonstrated that in that form duration of life is inherited in accordance with Mendelian principles (1, 2). In the case of man all the evidence so far available regarding the inheritance of duration of life has come from statistical studies, notably those of Pearson (3) and his associates, and Alexander Graham Bell (4, 5). This sort of evidence, while entirely cogent as to the main fact of the inheritance of this character, is not such as to make possible the ready determination of the mode of the inheritance, whether Mendelian or other.

Since the publication of my Lowell Lectures on the biology of death, I have received from a number of correspondents bits of data upon one or another phase of the problems therein discussed. Amongst these contributions was an extremely interesting one from a southern lady regarding the facts about duration of life in her own family. This note is based upon the information so received. For obvious reasons names cannot be given here, but I may say, as bearing upon the trustworthiness of the data here set forth, that my informant is a trained observer along biological lines, and is well known for her contributions in one field of biology.

The pedigree shown in Fig. 1 may be first presented, and then the probable meaning of the facts it shows, discussed. It should be noted that in this pedigree ♂ denotes as usual a male individual, ♀ a female, and ○ an individual known to have existed, but about whom I have no further information, even as to sex. The numbers standing above and to the right of each sex sign are the designatory numbers (in lieu of names) of persons included in the pedigree. The numbers

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* Papers from the Department of Biometry and Vital Statistics, School of Hygiene and Public Health, Johns Hopkins University, No. 47.

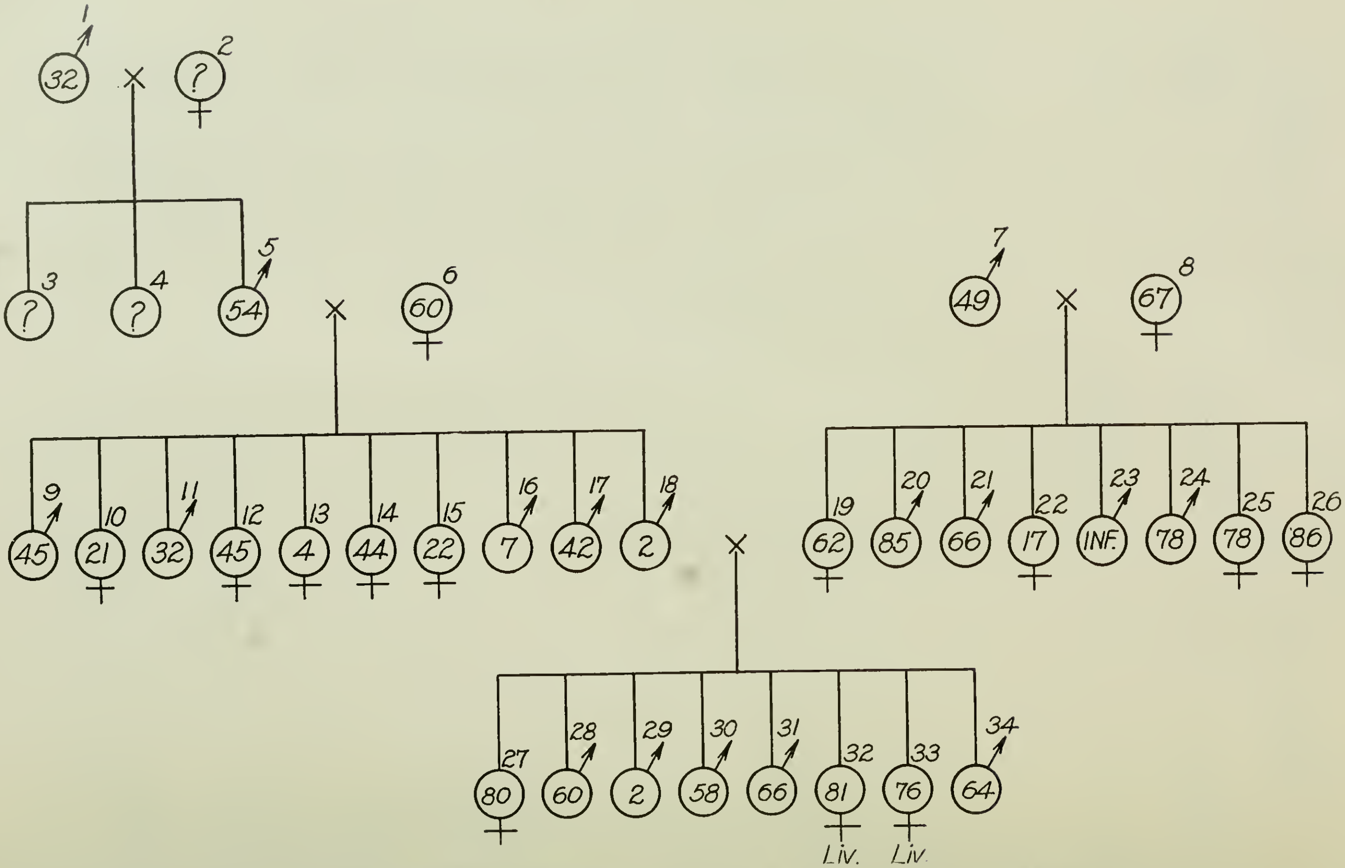

FIG. 1. Pedigree to show inheritance of duration of life in a southern family. For explanation see text.

within the circle of the sex sign give the ages at death of the individuals in years. All persons shown in the pedigree are dead with the exception of two marked "liv." below the numbers denoting their ages. One of these is my informant, to whom I am greatly indebted, both for furnishing me with the facts and for permitting their publication.

About individuals 3 and 4 no information is at hand. Their father died at the age of about 32 when they were small children. I furthermore have no information about the ancestry of individuals 2, 6, 7 and 8, except that individual 19 was reputed to "belong to a family with a remarkable record for longevity." The ages at death of both individuals 7 and 8 undoubtedly are under their potential longevity. No. 7's death "may be regarded as the nature of an accident, since he was a victim of yellow fever during the epidemic that visited Savannah in 1820." "His wife" (no. 8) "died at 67 of typhus fever contracted by remaining on the plantation to aid in caring for the sick negroes during a violent epidemic of the disease in 1852. As I remember her, she was a woman of wonderful energy and activity up to that time."

TABLE 1.

Survivorship of parents and offspring in pedigree Fig. 1.

Surviving up to	Number surviving to specified time are					
	A. Father's sibship (♂ P).		*B.* Mother's sibship (♀ P).		*C.* Offspring sibship (F₁).	
	Absolute.	Per cent.	Absolute.	Per cent.	Absolute.	Per cent.
Birth	10	100	8	100	8	100
10 Years	8	80	7	87.5	7	87.5
20 "	8	80	6	75	7	87.5
30 "	6	60	6	75	7	87.5
40 "	5	50	6	75	7	87.5
50 "	1	10	6	75	7	87.5
60 "	1	10	6	75	6	75
70 "	1	10	4	50	3	37.5
80 "	0	0	2	25	2	25
80 and over	0	0	2	25	2	25
Mean duration of life*	33.7 years		59.1 years		60.9 years	

* Counting the "infant" as aged 1, and taking the two living at present age.

The data of the pedigree are arranged in the form of survivorship figures in Table 1. Column *A* gives the survivorship relations of the family containing individuals 9 to 18 inclusive; Column *B* gives the same material for the family containing individuals 19 to 26 inclusive; and Column *C* the family containing individuals 27 to 34 inclusive. On account of the small numbers involved no attempt is made to smooth in any way the l_x lines. They are merely the observed frequencies of survival.

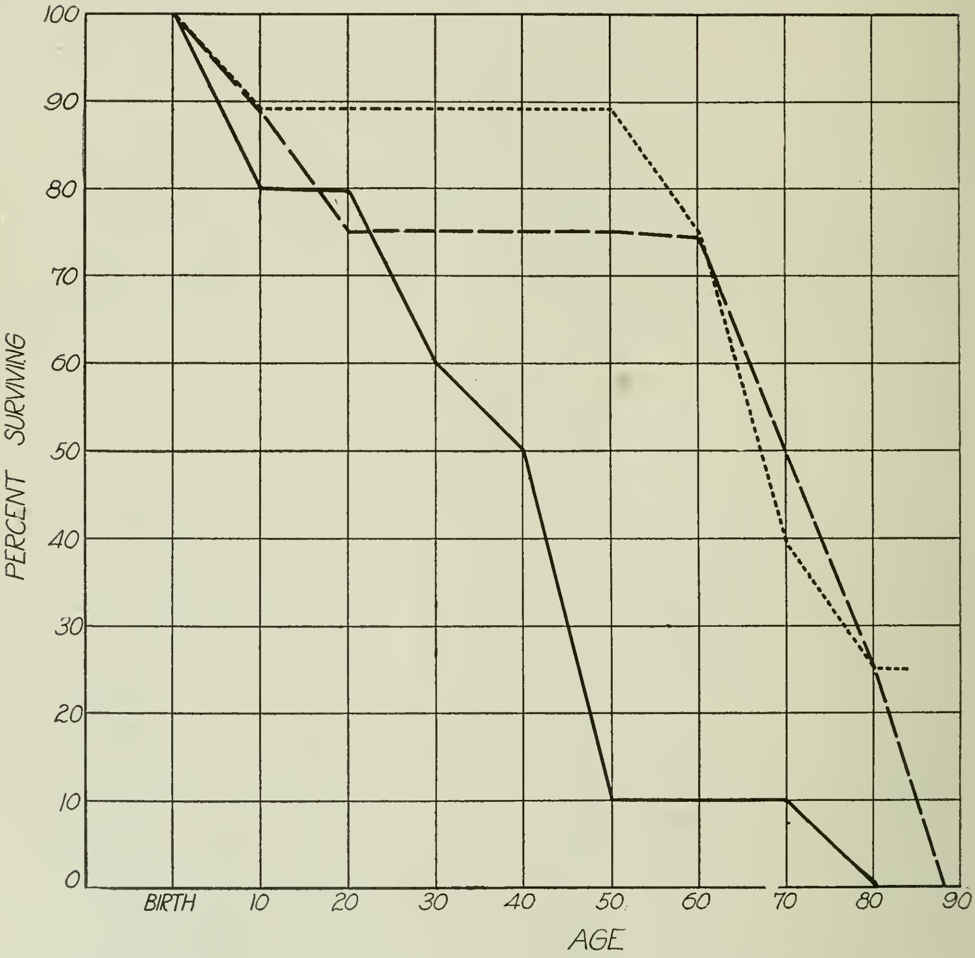

FIG. 2. Showing survivorship in three groups of sibs. The data of Column *A* of Table 1 are given as a solid line, those of Column *B* as a dash line, and those of Column *C* as a dot line.

The percentage columns of Table 1 are exhibited graphically in Fig. 2.

It is apparent from the table and diagram that the interest of this case lies in the fact that it represents the crossing of a short-lived with a fairly long-lived stock. In the father's sibship only 10 per cent. survived to age 50, in the mother's sibship 75 per cent. survived to that age, and in the offspring sibship 87.5 per cent.

Making due allowance for the irregularity of the survivorship lines of Fig. 2, consequent upon the small absolute numbers, the resemblance of this diagram to what one gets in a controlled Mendelian experiment on duration of life in *Drosophila* (cf. Fig. 4, p. 149, in my paper on the Biology of Death, VI, loc. cit.) is striking.

No conclusion can be drawn from a single pedigree of this sort, of course, but the case suggests the desirabality of analyzing extensive genealogical material in a similar manner. This I hope to be able to do later, upon material now accumulating in this laboratory.

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THE USE OF THE ORIGINAL DIAGNOSTIC CULTURE FOR THE DETERMINATION OF THE VIRULENCE OF DIPHTHERIA BACILLI.*

By LEON C. HAVENS, M.D., and HORACE M. POWELL.

(Received for publication January 3d, 1922.)

It is frequently desirable to know whether a throat culture, which is positive on the basis of morphology, actually contains toxin-producing diphtheria bacilli. This is particularly the case when one is dealing with convalescent patients and healthy carriers. Since the virulent and avirulent types of this organism cannot be distinguished by means of their cultural and morphological characteristics, it is necessary to resort to animal tests. The animal test most commonly used consists of giving a guinea pig a subcutaneous injection of a certain quantity of a broth culture, which has been inoculated from an isolated colony of a plate made from the diagnostic culture. As a rule a control pig, which has been given antitoxin, is given the same amount of culture as the test animal. The criterion for virulence is the death of the test pig or the nature of the local lesion. The procedure entails the isolation of pure cultures and the use of two animals for each culture tested. Aside from the time and expense involved, this method is not absolutely satisfactory in that the test culture comes from only one of many colonies and when a negative result is obtained one is faced with the possibility of having selected a colony of avirulent organisms while other colonies from the same diagnostic culture contained virulent organisms. It would be a decided advantage, therefore, to have a method which would reduce the items of time and expense, be more representative of the whole diagnostic culture and, at the same time, give accurate end results.

In 1915 Zingher and Soletsky (1) introduced the intracutaneous method of making diphtheria virulence tests. This method differs from the subcutaneous method in that the cultures, after isolation, are grown on solid medium, suspended in salt solution and injected into the skin of a guinea pig. Since the reaction (when the proper amount of culture is injected) is limited to a local skin lesion, from 4 to 6 cultures can be tested on one animal. This procedure reduces the expenditure for animals, but it is just as time consuming and is no more representative of the whole culture than the subcutaneous method.

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* From the Department of Immunology, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.

While engaged in a study of diphtheria which entailed the isolation of from 10 to 20 pure cultures daily, we received a personal communication from Dr. John N. Force, University of California, advocating the use of whole diagnostic cultures, instead of pure cultures, for making virulence tests by the intracutaneous method. We have made a series of tests with whole cultures, checking the results with pure cultures isolated from them, by both the intracutaneous* and the subcutaneous methods. A summary of the work is presented in the present paper.

Experimental.

The cultures.—The cultures, 509 in number, were from patients and contacts—diagnostic and release cultures—and from healthy individuals not associated with cases. The cultures were collected during the different seasons of the year in order that the contaminating organisms might be as widely varied as possible. The cultures were made on Loeffler's serum medium and incubated from 12 to 18 hours.

Preparation of the cultures for injection. After the microscopic examination, the culture was washed from the medium with 1 or 2 c.c. of sterile salt solution and transferred to a clean sterile tube and further diluted as desired. The secondary dilution was roughly proportional to the relative number of diphtheria bacilli present, more salt solution being added to the cultures which contained numerous bacilli than to those which contained only a few. The heaviest cultures were suspended in 5 or 6 c.c. of salt solution.

The injection. It is advisable to use guinea pigs weighing more than 400 grams because a larger number of tests can be made on one animal and the lesions do not spread as readily as they do in smaller animals. The hair is removed from the ventral surface by plucking and carefully shaving off the short, fine hairs which the plucking leaves. Six cultures can be satisfactorily tested on the largest pigs and four on smaller ones. 0.1 c.c. of each suspension is injected into the skin, the different injections being as widely separated as possible. The same suspensions are injected at corresponding points in a second pig which has been given 250 units of antitoxin 24 hours previously. This control is absolutely necessary.

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* Before beginning the work with whole cultures, 677 pure cultures had been tested for virulence by the intracutaneous method. More than 100 of these tests were checked by the subcutaneous method. The intracutaneous method proved to be quite satisfactory, when the proper care was used in regard to the number of bacilli injected. This was controlled by diluting the suspensions to a standard opacity, about 20 million organisms being injected.

Interpretation of the reaction. The guinea pigs should be inspected 24, 48 and 72 hours after the injections are made, the lesions on the test animals being compared with the corresponding ones on the controls. There is but one dependable criterion for virulence, viz.: necrosis of the superficial layers of the skin of the test pig, while it is absent in the control. The control pig may show as much edema and discoloration as the normal, but there is no necrosis or sloughing, even when the contaminating staphylococci, streptococci, etc., cause abscess formation. The size of the lesion has little significance. A very small lesion showing typical necrosis indicates that toxin producing bacilli were injected, while an extensive lesion without necrosis is negative with respect to virulent diphtheria bacilli. It is advisable to examine the lesions carefully each day, recording the observations made, but not to record them as definitely positive or negative until the third day, unless characteristic necrosis appears earlier.

Results. Of the 509 whole cultures, tested as just described, 341 were positive on microscopic examination and 168 negative. None of the microscopically negative cultures gave positive virulence tests, while 305 of the microscopically positive cultures gave positive virulence tests, and 36 negative tests.

Pure culture tests. Pure cultures were isolated from all but 46, or 295, of the microscopically positive cultures used in the study, and were tested for virulence by the intracutaneous method. A small number of the cultures which gave doubtful results by this method were retested by the subcutaneous method. 263 of the 295 pure cultures gave positive tests and 32 negative tests.

It is seen that 295 cultures were tested both by the whole-culture and the pure-culture methods. 275, about 93 per cent., of the individual cultures gave the same result by both methods. There were, then, 20 discrepancies. Eight of these were positive with the whole culture and negative with the pure culture; 12 negative with the whole culture and positive with the pure culture. This shows that the whole-culture method gave a negative result with 12 cultures (4 per cent.) which actually contained virulent diphtheria bacilli. This was probably due to the small number of diphtheria bacilli present. The 8 cultures that were positive by the whole-culture method, while the pure cultures isolated from them gave negative tests, cannot be counted against the whole-culture method, since it is possible that the cultures contained both virulent and non-virulent bacilli, the pure cultures being obtained from colonies of the latter. If this be true, it is a point in favor of the whole-culture test. This point will be taken up again in a subsequent paragraph.

As stated above, pure cultures were not isolated from 46 of the cultures (13 per cent. of the microscopically positive cultures) which gave positive tests by the whole-culture method. These cultures would not have been tested at all had we used only the pure-culture method. This percentage might have been reduced by an improvement in the technique of isolation, but there would still be a considerable percentage of microscopically positive cultures from which pure cultures could not be obtained and hence not tested for virulence. These cultures can be tested by the whole-culture method.

Virulent and avirulent strains from the same culture. In order to determine the frequency of the co-existence of virulent and avirulent diphtheria bacilli in throat cultures and the probability of selecting avirulent colonies when both virulent and avirulent strains are present, 17 cultures were selected and from 2 to 33 colonies were fished from the plates made from the individual cultures. Each culture thus obtained was tested for virulence. The results are given in Table 1.

As seen from Table 1, a total of 210 colonies were fished from the 17 cultures. 205 or 97.6 per cent. of these were found to contain virulent organisms. It is significant that 4 of the 17 cultures contained avirulent organisms, the numerical relation of virulent and avirulent colonies being respectively 32 to 1, 13 to 1, 8 to 1, and 11 to 1. Hence the possibility of error when only one colony is used to represent the whole culture in the virulence test is not inconsiderable, and it supports our contention that this may be the explanation of the discrepancies between the whole- and pure-culture tests in the case of the 8 instances where the whole-culture test was positive while the pure-culture test was negative.

Artificially mixed cultures. It now became of interest to know what influence the most common contaminants had upon the outcome of the whole-culture virulence tests. For this purpose the lowest number of organisms which would give a positive virulence test was determined for a given strain of *B. diphtheriae.* A known number

TABLE 1.

Virulent and avirulent diphtheria bacilli from the same throat culture.

Cultures.	Number of colonies picked.	Number of colonies giving virulent cultures.	Number of colonies giving avirulent cultures.
147	33	32	1
153	8	8	0
154	7	7	0
155	12	12	0
157	2	2	0
158	20	20	0
160	14	13	1
161	18	16	2
162	12	12	0
164	13	13	0
182	16	16	0
185	14	14	0
189	12	11	1
190	2	2	0
191	18	18	0
204	3	3	0
244	6	6	0
Total 17	210	205	5

of staphylococci or streptococci which had been isolated from throat cultures were mixed with " sub-positive " numbers of the diphtheria bacilli and an intracutaneous virulence test made with the mixture. The results are summarized in Table 2.

TABLE 2.

Intracutaneous virulence tests with artificially mixed cultures.

Number of diphtheria bacilli.	Number of staphylococci.	Number of streptococci.	Result.
400,000	0	0	+
300,000	0	0	−
200,000	0	0	−
300,000	300,000	0	+
200,000	200,000	0	+
200,000	20,000,000	0	+
300,000	0	300,000	+
200,000	0	200,000	+
200,000	0	20,000,000	+

It is seen from Table 2 that 400,000 diphtheria bacilli in pure culture gave a positive test, while 200,000 and 300,000 failed to produce a characteristic lesion. However, when mixed with an equal number of staphylococci or streptococci 200,000 diphtheria bacilli were sufficient to cause a positive lesion. It is further seen that the diphtheria bacilli produce a characteristic lesion even when there are 100 times as many staphylococci or streptococci as diphtheria bacilli present. The apparent enhancement of the virulence of the diphtheria bacilli by the staphylococci or streptococci is probably due to the fact that the destruction of the bacilli is retarded, giving them a longer time in which to produce toxin. It is less probable that the cocci render the tissues of the guinea pig more susceptible to the necrotizing action of the toxin.

Conclusions.

1. The original diagnostic culture can be used advantageously for the diphtheria bacillus virulence test by the intracutaneous method.
2. The method eliminates the necessity of isolating pure cultures and makes it possible to test any desired number of cultures.
3. The results obtained by this method check with those obtained by the pure culture methods, and, moreover, it is possible to determine the virulence of cultures when pure cultures cannot be isolated.

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NOTE ON CERTAIN CORRELATION FACTORS OF THE 1918 INFLUENZA EPIDEMIC.*

By C.-E. A. WINSLOW

AND

C. C. GROVE.

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In his first statistical study of the 1918 epidemic of influenza, Raymond Pearl (1) showed that the explosiveness of the outbreak in a group of the larger American cities was strikingly correlated with the normal death rate from all causes in those cities during the year 1916, and particularly with the death rates from pulmonary tuberculosis, diseases of the heart and diseases of the kidneys. In the following year Winslow and Rogers (2) obtained a similar correlation for 94 towns in Connecticut between the severity of the influenza epidemic and the death rate from all causes during the period, 1915–17. It was found in the latter study that the severity of the epidemic was also apparently correlated with the date at which it reached its climax in a given community, and the suggestion was made that the relationship between the incidence of the influenza epidemic and the previous normal death rates might be either a direct or an indirect one. " The higher pneumonia rates and total death rates in the eastern section may reasonably be attributed to unfavorable economic conditions, associated with industrial life and presence of certain foreign race stocks, always characterized by a high death rate. Whether the greater severity of the influenza epidemic in these same cities was due to the same causes or to a gradual loss of communicability or virulence, as the infection passed westward from its point of introduction, seems uncertain."

Last spring Dr. Pearl (3) published a more exhaustive study of the statistics of the influenza epidemic in the larger cities of the United States, with computations of partial correlations. In this study it was shown that the explosiveness of the influenza outbreak was not correlated with the age or sex distribution of the population, with the density or rate of recent growth of the population, or with the longitude of the city, but was correlated to a slight degree with latitude (— 0.376 ± 0.099) (4) and was highly correlated with the previous death-rates from all causes (+ 0.572 ± 0.078) (5) and from organic diseases of the heart (+ 0.562 ± 0.079) (6), when all the conditions previously cited were held constant. Previous death rates from cancer and typhoid fever showed no correlation, and those from pulmonary tuberculosis and acute nephritis and Bright's disease a slight correlation. Correlations for the destructiveness of the outbreak (excess mortality for the 25-week period) showed results almost identical with those obtained for explosiveness.

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* From the Department of Public Health of the Yale University School of Medicine, New Haven, Conn., and the Department of Mathematical Statistical Research of the Metropolitan Life Insurance Co.

In view of certain broad epidemiological observations which suggested that the severity of the influenza epidemic might probably be related to cyclical fluctuations in the virulence of the causative organism we were not quite satisfied that such an influence was excluded by Pearl's studies. Neither latitude nor longitude gives a direct measure of the time-relations of the outbreak, for obvious reasons, and we felt that it would be worth while to introduce the time-relation itself into the problem, and determine its influence upon the other factors which Pearl had shown to be important. The peak week of the epidemic in the group of cities studied varied from the week ending October 5, 1918 (Boston and Cambridge), to the week ending December 14, 1918 (Grand Rapids, Milwaukee and St. Louis). The weeks included in this period were numbered from 1 to 11, and the resulting numbers taken as a measure of the time-relations of the outbreak.

The fundamental data used in the present study are presented in Table 1. Columns *A*, *B* and *C* are taken directly from Pearl's last paper (7), and refer respectively to the explosiveness of the influenza epidemic (as measured by Pearl's index I_6), to the death rate from all causes in the years 1915–17, and to the latitude. Column *D* contains the new figures for time-relation.

Table 2 shows the deviations from the means for each of the columns in Table 1.

With these data we have obtained the following primary correlations.

TABLE 1.

Data for correlation of characteristics of cities with explosiveness of epidemic influenza mortality.

City.	*A**	*B*†	*C*‡	*D*§
Albany, N. Y.	32.2	1,947.0	42.65°	4
Atlanta, Ga.	5.3	1,551.2	33.73	4
Baltimore, Md.	43.4	1,810.3	39.28	3
Boston, Mass.	28.8	1,651.6	42.36	1
Buffalo, N. Y.	21.1	1,566.4	42.88	4
Cambridge, Mass.	17.9	1,354.4	42.38	1
Chicago, Ill.	13.2	1,457.1	41.88	4
Cincinnati, O.	9.2	1,618.0	38.14	4
Cleveland, O.	17.7	1,462.1	41.50	5
Columbus, O.	11.0	1,498.3	40.00	4
Dayton, O.	28.8	1,492.7	39.73	3
Fall River, Mass.	27.8	1,639.4	41.70	2
Grand Rapids, Mich.	2.5	1,258.8	42.97	11
Indianapolis, Ind.	8.6	1,550.2	39.67	3
Los Angeles, Calif.	10.0	1,238.2	34.08	5
Louisville, Ky.	21.5	1,542.1	38.20	3
Lowell, Mass.	24.7	1,681.9	42.65	2
Milwaukee, Wis.	4.9	1,214.4	43.05	11
Minneapolis, Minn.	4.1	1,189.4	44.97	4
Nashville, Tenn.	41.5	1,683.7	36.15	3
Newark, N. J.	14.1	1,412.2	40.75	4
New Haven, Conn.	11.1	1,660.0	41.32	4
New Orleans, La.	34.1	1,983.0	29.97	4
New York, N. Y.	12.8	1,384.7	40.71	4
Oakland, Calif.	15.1	1,082.8	37.75	5
Philadelphia, Pa.	47.9	1,630.1	39.95	3
Pittsburgh, Pa.	21.3	1,694.0	40.43	5
Providence, R. I.	14.0	1,527.0	41.84	3
Rochester, N. Y.	13.9	1,456.8	43.13	4
St. Louis, Mo.	5.4	1,458.0	38.63	11
St. Paul, Minn.	4.5	1,093.0	44.87	7
San Francisco, Cal.	25.4	1,546.5	37.79	5
Toledo, Ohio	17.9	1,705.1	41.77	4
Washington, D. C.	30.7	1,795.6	38.89	3
Average	18.894	1,524.588	40.170	4.3235

* *A*. Explosiveness of outbreak of epidemic mortality as measured by an epidemicity index I_6. Column 1 in Pearl's table.

† *B*. Normal death rate from all causes, 1915–1917. Column 3*f* of Pearl's table.

‡ *C*. Latitude. Column 7 of Pearl's table.

§ *D*. Week of highest peak.

TABLE 2.

Deviations from means of data presented in table 1.

City.	x_1 (*A*).	x_2 (*B*).	x_3 (*C*).	x_4 (*D*).
Albany, N. Y.	+13.306	+422.412	+2.480	−0.3235
Atlanta, Ga.	−13.594	+26.612	−6.440	−0.3235
Baltimore, Md.	+24.506	+285.712	−0.890	−1.3235
Boston, Mass.	+9.906	+127.012	+2.190	−3.3235
Buffalo, N. Y.	+2.206	+41.812	+2.710	−0.3235
Cambridge, Mass.	−.994	−170.188	+2.210	−3.3235
Chicago, Ill.	−5.694	−67.488	+1.710	−0.3235
Cincinnati, O.	−9.694	+93.412	−2.030	−0.3235
Cleveland, O.	−1.194	−62.488	+1.330	+0.6765
Columbus, O.	−7.894	−26.288	−0.170	−0.3235
Dayton, O.	+9.906	−31.888	−0.440	−1.3235
Fall River, Mass.	+8.906	+114.812	+1.530	−2.3235
Grand Rapids, Mich.	−16.394	−265.788	+2.800	+6.6765
Indianapolis, Ind.	−10.294	+25.612	−0.500	−1.3235
Los Angeles, Calif.	−8.894	−286.388	−6.090	+0.6765
Louisville, Ky.	+2.606	+17.512	−1.970	−1.3235
Lowell, Mass.	+5.806	+157.312	+2.480	−2.3235
Milwaukee, Wis.	−13.994	−310.188	+2.880	+6.6765
Minneapolis, Minn.	−14.794	−335.188	+4.800	−0.3235
Nashville, Tenn.	+22.606	+159.112	−4.020	−1.3235
Newark, N. J.	−4.794	−112.388	+0.580	−0.3235
New Haven, Conn.	−7.794	+135.412	+1.150	−0.3235
New Orleans, La.	+15.206	+458.412	−10.200	−0.3235
New York, N. Y.	−6.094	−139.888	+0.540	−0.3235
Oakland, Calif.	−3.794	−441.788	−2.420	+0.6765
Philadelphia, Pa.	+29.006	+105.512	−0.220	−1.3235
Pittsburgh, Pa.	+2.406	+169.412	+0.260	+0.6765
Providence, R. I.	−4.894	+2.412	+1.670	−1.3235
Rochester, N. Y.	−4.994	−67.788	+2.960	−0.3235
St. Louis, Mo.	−13.494	−66.588	−1.540	+6.6765
St. Paul, Minn.	−14.394	−431.588	+4.700	+2.6765
San Francisco, Calif.	+6.506	+21.912	−2.380	+0.6765
Toledo, Ohio	−.994	+180.512	+1.600	−0.3235
Washington, D. C.	+11.806	+271.012	−1.280	−1.3235

Correlation between

r_{12} Influenza explosiveness and normal death rate from all causes, + 0.6781 ± 0.0625

r_{13} Influenza explosiveness and latitude .... — 0.2426 ± 0.1089

r_{14} Influenza explosiveness and date of peak week .... — 0.5243 ± 0.0839

r_{23} Normal death rate from all causes and latitude .... — 0.3131 ± 0.1043

r_{24} Normal death rate from all causes and date of peak week — 0.4437 ± 0.0929

r_{34} Latitude and date of peak week .... + 0.0795 ± 0.1149

The correlation between epidemic explosiveness and normal death rate from all causes is identical with that obtained by Pearl and is very high. The correlation between epidemic explosiveness and latitude also checks that obtained by Pearl (— 0.243 ± 0.109). It is of doubtful significance since the correlation is less than 3 times its probable error. The odds are only 6.5 to 1 against such a deviation occurring in simple sampling at any one trial. The new correlation between explosiveness and date of peak week (— 0.5243 ± .0839) is high.

Passing to partial correlations, however, we find somewhat different results as follows.

	Correlation between	Factors held constant	Correlation
$r_{12.34}$	Influenza explosiveness and normal death rate from all causes.	Latitude and date of peak week.	0.5523 ± 0.0804
$r_{13.24}$	Influenza explosiveness and latitude.	Normal death rate and date of peak week.	0.0714 ± 0.1151
$r_{14.23}$	Influenza explosiveness and date of peak week.	Normal death rate and latitude.	0.3426 ± 0.1021

Total or Multiple Coefficient of Correlation $r_{1-234} = 0.7241 \pm 0.0540$.

When analyzed in this way the correlation between the explosiveness of the epidemic and the normal mortality from all causes remains substantially unaffected, being still over 0.5 and about seven times its probable error. The correlation of explosiveness with latitude disappears entirely, being apparently of no significance. The correlation of explosiveness with the date of the peak week is also substantially reduced by holding the normal death rate and latitude constant, —reduced to such a degree that, although it remains three times the probable error, its significance must be considered to be doubtful.

We conclude therefore that the relation between the explosiveness of the influenza epidemic and the date at which it reached its maximum in a given community is not a clear or a striking one, as we had expected it might prove to be. Pearl's discovery of the relation which the explosiveness of the epidemic bore to the normal death rate from all causes and from organic heart disease in the affected communities remains perhaps the most outstanding discovery which has been made in the study of this disease since the recent outbreak. Such a correlation, as he has demonstrated, cannot possibly be due to fortuitous circumstance. It indicates a real relation between the two factors studied. It is still important, however, to remember that a high statistical correlation may indicate either a direct or an indirect relationship. We cannot say (as some may perhaps be tempted to do) that the influenza outbreak was severe in a given community, *because* the death rate from organic heart disease had previously been high in that community. Such may have been the case; but it is equally possible that the high mortality from organic heart disease and the explosiveness of the influenza epidemic, manifest in the same communities, may both have been due to some common cause or causes affecting both, such as the racial composition of the population, or the prevalence of certain other pathogenic micro-organisms in the community. The fact of the relationship between the explosiveness (and also between the destructiveness) of the influenza epidemic and the previous normal death rate, from all causes and from organic diseases of the heart, is definite and most important. Its explanation is still problematical.

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ON THE PRODUCTION OF HEMOLYSINS AND HEMAGGLUTININS IN THE DOMESTIC FOWL.*

By ROSCOE R. HYDE, Ph.D., and CLENNIE E. BAILEY.

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The production of immune hemolytic sera was first demonstrated by Bordet (1) in 1898. He injected rabbit blood into the guinea pig and made the far-reaching observation that the serum from the treated animal became powerfully lytic for rabbit corpuscles. Subsequent observations on different species of animals and with many different kinds of red corpuscles have shown that Bordet's discovery holds as a general principle in immunological reactions, i.e., the serum of an animal that has been treated with the red corpuscles of another species becomes powerfully lytic for these cells. The acquired lytic property is due to an increase in the thermostabile amboceptor or sensitizer content of the serum. It is equally true, on the other hand, that all animal species do not react equally to immunization with the corpuscles of a particular species, and that a given species does not react in the same manner to different kinds of corpuscles. For example, the cat and the dog respond very poorly, compared with the rabbit, to intraperitoneal injections of sheep corpuscles. The rabbit yields a most potent sensitizer for sheep corpuscles but produces a less potent serum for human corpuscles. Very potent hemagglutinating and hemolytic serum is obtained from the rabbit by intravenous injections of fowl cells, while with ox cells one obtains a serum of high hemolytic titer, but the agglutinating titer is practically nil. Further examples might be cited to show that it is not possible to predict the result of immunizing a given species with a particular kind of cell and that each case must be determined by trial.

The present paper deals with the production of hemolysins and hemagglutinins in the domestic fowl. Citron (2) specifically mentions the domestic fowl as one of the species most suitable for the production of hemolytic sera. In his "Immunity" (Garbat translation, 1912, page 131) the following statement is found: "As far as the technique for obtaining immune-hemolysins is concerned, the rules which hold for every process of immunization are naturally to be followed here also. It is not possible, however, to immunize every kind of animal against every type of blood corpuscle. Rabbits, goats, horses, and chickens are the ones which are best adapted to supply hemolytic sera. An animal produces a better hemolysin the remoter its relationship to the animal from which the erythrocytes for injection are taken.'' No evidence is given to justify the inclusion of the domestic fowl among the species best adapted to produce hemolytic sera, and, so far as is known to me, none exists. In spite of this fact Citron's statement is still taken at its face value. Thus, Guyer and Smith (3) have recently made use of it in support of their contention of having produced in the chicken a serum lytic for the eye lens of the rabbit, with which results of great biological significance were obtained. The serum of chickens which had been treated with rabbit lens was injected into the circulation of pregnant rabbits. Some of the young of these rabbits had an eye defect which was passed on to succeeding generations. It was contended that the eye defect was in all probability due to the cytolytic action of the chicken serum, since chickens were known to be good cytolysin (hemolysin) producers.

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* From the Department of Immunology, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.

Before taking up the experimental work it is necessary to point out that chicken serum is distinctly peculiar with respect to the reactivation of its hemolytic action. As a rule, sera containing either native or acquired hemolysins can readily be reactivated with fresh serum from the same or other species. Heated or aged chicken serum is an exception to this rule. Reactivation does not occur when the corpuscles, sensitizer, and complement are mixed in the usual way. It is necessary to mix and incubate the sensitizer and complement, before the corpuscles are added, if hemolysis is to be effected (4).

EXPERIMENTAL

Domestic fowls were treated over varying periods of time with the blood corpuscles of the following species: pigeon, sheep, guinea pig, ox, and rabbit. The agglutinating and hemolytic titers of the serum were determined both before and during the course of the treatment. A record of the titration, the kind of corpuscles used, the routes of injection, etc., is given in connection with each individual case.

General technique. Fresh corpuscles were used both for the injections and titrations. The blood was collected in a solution of sodium citrate, the corpuscles sedimented by centrifugalization, washed four times in large volumes of salt solution, and finally enough salt solution was added to make the volume the same as that of the whole blood. Any further dilution is indicated in the individual protocols. The chicken serum was obtained by collecting the blood from the wing vein and centrifugalizing it after clotting had occurred.

The hemolytic power of the fresh serum was determined without the addition of foreign serum as complement. This will be spoken of as the " active " hemolytic titer. The test was made by putting varying quantities of undiluted serum and of $\frac{1}{10}$ or $\frac{1}{100}$ dilutions in a series of serological tubes, bringing the volume up to 0.9 c.c. with salt solution and then adding 0.1 c.c. of a $\frac{1}{4}$ dilution of corpuscles. The tubes were thoroughly shaken and put in the water bath (37° C.) for one hour. Readings were made at the end of the incubation period. The degree of hemolysis was noted and indicated in the records by numbers, 4 signifying complete, 1- a trace of hemolysis, and 3, 2 and 1 intermediate degrees. The agglutinating power of the sera was determined in a similar manner, after inactivation at 55° C. for 30 minutes.

During the first part of the work we were unable to reactivate the hemolytic property of the serum after it had been heated at 55° C. for 30 minutes. A number of fresh sera were used as complement, and our results were uniformly negative. It was found later that the natural hemolysins for rabbit cells could be reactivated after inactivation at 55° C. with either fresh guinea pig or fresh chicken serum. This point will be considered more fully in connection with the protocols in which it was employed.

Experiment 1 (Rabbit corpuscles). A large Plymouth Rock hen, whose serum had an " active " hemolytic titer of $\frac{1}{35}$, was given first, 15 subcutaneous, and then 2 intravenous injections of rabbit corpuscles during the course of three months. The injections were made at from 2 to 7 day intervals, a total of 62.5 c.c. of corpuscles being given. The second intravenous injection (2 c.c. of corpuscular suspension) caused a severe reaction, simulating an anaphylactic shock. The hen was found dead one week later. The serum was tested for both hemolysins and hemagglutinins at six different times during the course of the treatment. The hemolytic titer was not raised. In fact, on two occasions it was lower than the original titer. The agglutinins showed a slight increase on the last test, the titer being $\frac{1}{500}$, while it had previously been $\frac{1}{400}$.

Experiment 2 (Rabbit corpuscles). A large male fowl was given

3 c.c. of rabbit cells intravenously. No perceptible reaction followed this injection. Three days later a similar injection was followed immediately by labored breathing and loss of power of locomotion. Two days after the second injection 4 c.c. of corpuscles were given intravenously. Within less than two minutes the rooster staggered and fell to the floor. Breathing became increasingly difficult and finally ceased in desperate efforts to take in air. Death ensued five minutes after the injection was made.

Experiment 3 (Sheep corpuscles). A large Plymouth Rock hen was given 11 subcutaneous and 2 intravenous injections of sheep corpuscles. The injections were made at from 3 to 8 day intervals during the course of two and a half months, a total of 43 c.c. of cell suspension being given. The last two injections were given intravenously. The intravenous injection of 3 c.c. of cells was followed by a severe reaction from which the hen soon recovered. The second intravenous injection caused an immediate anaphylactic-like reaction and death.

The serum of this chicken was tested for hemolytic power at different times during the course of the injections. The titer was only $1/10$ one week after the last subcutaneous injection was made.

Experiment 4 (Sheep corpuscles). The " active " hemolytic titer of the serum of the hen used in this experiment was $1/10$ and the agglutinating titer $1/33$. She was given 11 intraperitoneal injections of sheep corpuscles during six weeks time. An average of 15 c.c. of cell suspension was given at each injection. The serum was retested six times after the injections were begun. Four weeks after the first injection, four injections having been given, the hemolytic titer was $1/50$ and the agglutinin titer $1/100$. Both titers subsequently became weaker, and at the last test were only $1/10$.

Further immunization experiments, similar to those given above, were carried out in which ox, guinea pig, and pigeon erythrocytes were used as antigens. Subcutaneous, intraperitoneal and intravenous injections were made during long periods of time. The results were not materially different from those already given. There was never more than a slight increase in the hemolytic power of the sera, and the agglutinating titers did not increase more than two- or four-fold. These increases came after the third or fourth injection, and were followed by a return to the original titers.

It may be argued that the method which we have used to determine the hemolytic power of the sera of the treated animals was faulty, since, in the dilution of the sera the complement was soon reduced to the minimum requirement of this factor, and that hemolysis failed to occur in higher dilutions because of a deficiency of complement and not because the hemolytic sensitizer was not increased as a result of immunization. That such was not the case finds support in the well-known fact that when the hemolytic amboceptor is increased less complement is required to effect a given amount of hemolysis. Thus, according to Noguchi, the amount of complement necessary to hemolyse one unit of red cells is ⅓, ⅕ and ⅒ of a unit when, 4, 10 and 20 units respectively of amboceptor are used. Hence a four-fold increase in the sensitizer content of a fresh serum should raise the titer three-fold; a ten-fold increase, five-fold; and a twenty-fold increase, ten-fold. Hence, the conclusion that the hemolysin content of the sera of the fowls was not greatly increased as a result of the extensive treatment with foreign red cells would seem to be justified.

When the work had progressed to this point it was found that the natural hemolysin in chicken serum for rabbit corpuscles could be reactivated with either fresh guinea pig or chicken serum, after being heated at 55° C. for 30 minutes. Consequently two other fowls were given a series of injections of rabbit cells and the sera titrated both before and after inactivation. The titrations with foreign complement were carried out as follows: The serum from normal or immunized fowls was heated in 5 c.c. quantities at 55° C. for 30 minutes. Graded quantities of the whole serum, or of suitable dilutions of the serum, were put into a series of tubes, and salt solution was added to bring the volume in each tube up to 0.8 c.c. A subhemolytic quantity of fresh chicken or guinea pig serum in a volume of 0.1 c.c. was added to each tube. The tubes were shaken and put in the water bath (37° C.) for 30 minutes. 0.1 c.c. of a ¼ dilution of rabbit erythrocytes was now put in each tube. The tubes were put in the water bath again for one hour. By this method the hemolytic power of heated normal chicken serum can be completely restored, while if the corpuscles, heated serum and fresh serum were put into the tubes at the same time, or if the heated serum and corpuscles were mixed before the fresh serum was added, no hemolysis occurred. The heated serum and the fresh serum must be mixed and incubated before the corpuscles are added.

Experiment 5 (Rabbit corpuscles). The serum of the fowl used in this experiment had an " active " hemolytic titer of $\frac{1}{25}$ for rabbit erythrocytes, and the heated serum (55° C. for 30 minutes) agglutinated the cells in a dilution of $\frac{1}{250}$. The inactivated serum plus a subhemolytic dose (0.02 c.c.) of fresh chicken serum gave a hemolytic titer of $\frac{1}{25}$. The fowl was then given four intraperitoneal injections of 3.5 c.c. each of rabbit corpuscles, the injections being given at two-day intervals. Six days after the last injection the serum gave an active titer of $\frac{1}{50}$. The titer of the inactivated serum, using chicken serum as the source of complement, was between $\frac{1}{25}$ and $\frac{1}{50}$. When fresh guinea pig serum was used as complement the titer was $\frac{1}{5}$. The hemagglutinins were not materially increased. It is evident that treatment had little influence upon the sensitizer and agglutinin content of the serum.

Experiment 6 (Rabbit corpuscles). A large Plymouth Rock rooster was given eight injections of rabbit erythrocytes during the course of eleven days, a total of 25 c.c. of cell suspension being given. The cells were injected both intravenously and into the peritoneum. The first treatment consisted of an intravenous injection of 4 c.c. of cell suspension. Two days later 2 c.c. of cells were injected into the peritoneal cavity. Three further treatments were given at three-day intervals. These consisted of an intraperitoneal injection of from 2 to 4 c.c. of cells and, three hours later, an intravenous injection of an equal amount. This method of treatment was adopted because of the severe and often fatal anaphylactic-like shocks that usually follow the intravenous injection of cells in fowls which have had one or more previous injections. This particular fowl survived the treatment, but the desensitizing action of the intraperitoneal injections was not entirely effective, since rather severe reactions always followed the intravenous injections.

Before treatment was begun the serum of this fowl had a hemolytic titer of $\frac{1}{33}$ and an agglutinating titer of $\frac{1}{250}$. Six days after the last treatment the titers were $\frac{1}{500}$ and $\frac{1}{1000}$ respectively. Subhemolytic doses (0.02 c.c.) of fresh chicken serum were used for complement in the hemolytic reactions. It is seen, therefore, that in this instance the potency of the serum was definitely raised as a result of the treatment. A similar result was not obtained in any other case.

Rabbit Erythrocytes Rendered Non-Antigenic by Chicken Serum.

The failure of the domestic fowl to respond energetically to immunization with foreign erythrocytes raises a question of general interest. Is the failure due to an absence of the proper receptors or affinities in the tissues of this animal, or may it not be due to the fact that the body fluids render the foreign cells non-antigenic before they can reach or stimulate the proper tissues of the host? The energetic way in which chicken serum acts upon the red blood cells of the rabbit in vitro suggested the latter as a possibility and experiments were planned to test this point.

Experiment 7. Five guinea pigs were given respectively three intraperitoneal injections, at 4-day intervals, of the following mixtures: Guinea pig 1, 1 c.c. of rabbit corpuscles in 8 c.c. of fresh chicken serum; Guinea pig 2, 1 c.c. of rabbit corpuscles in 8 c.c. of distilled water; Guinea pig 3, 1 c.c. of rabbit corpuscles in 8 c.c. of salt solution; Guinea pig 4, 8 c.c. of chicken serum; Guinea pig 5, 1 c.c. of sheep corpuscles in 8 c.c. of fresh rabbit serum. The mixtures were allowed to stand at room temperature for twelve hours, and were then put at 37° C. for 3 hours before they were injected. This procedure was repeated three times. Seven days after the last injection the guinea pigs were bled and their sera were tested for hemolysins in the usual way. The results were as follows: Guinea pig 1, no hemolysins for rabbit cells; Guinea pig 2, hemolytic titer for rabbit cells, 1–1250; Guinea pig 3, hemolytic titer for rabbit cells, 1–1250; Guinea pig 4, no hemolysins for rabbit cells; Guinea pig 5, hemolytic titer for sheep cells, 1–2000. Experiment 7 was repeated four times with practically the same results. It is seen that rabbit red blood cells hemolysed with fresh chicken serum no longer cause the formation of specific hemolysins in the guinea pig, while water hemolysis does not alter the antigenic properties of the cells. It is further seen that hemolysis by serum does not necessarily destroy the antigenic properties of the hemolysed cells, for sheep cells hemolysed with fresh rabbit serum still engendered the formation of specific antibodies when injected into guinea pigs.

Discussion and Summary.

Normal chicken serum lyses and agglutinates the erythrocytes of many other species. The lytic property depends upon two factors, one thermostabile, the other thermolabile. When the serum becomes inactive as a result of exposure to heat or through aging, the lytic property is not restored by the addition of fresh serum from other species to the usual hemolytic tests. Reactivation can be effected, however, by mixing and incubating the heated and fresh serum before the corpuscles are added.

The foregoing work shows that chickens do not respond to treatment with foreign red blood cells as most other species do. In only one instance was there a material increase in the lytic power of the serum. This peculiarity on the part of the chicken is of interest in connection with the theories of the origin and nature of antibodies, and a knowledge of the mechanism here involved may aid in understanding failures to stimulate antibody formation under other conditions.

If antibodies are cast-off receptors, the tissues of the chicken should be well supplied with receptors for rabbit corpuscles, because we find numerous free receptors in the blood stream. Antigenic stimulation should give rise to increased production of receptors, and the serum should become more potent as the result of an accumulation of free receptors or antibodies. It is evident that the theory is wrong or that the injected cells or their constituents do not reach the cell receptors and stimulate the formation of an excess of receptors and thus new antibodies. Since it has been shown that fresh chicken serum acts upon rabbit blood cells in vitro in such a way as to render the cells non-antigenic for guinea pigs, it seems probable that a similar thing occurs in vivo and that the foreign cells are rendered non-antigenic, before the tissues of the host have been sufficiently stimulated. A similar condition may obtain in other instances where animals do not respond to immunization.

In the light of our observations on the production of hemolysins in the chicken, it seems improbable that Guyer and Smith (3) produced in this species a serum lytic for the eye lens of the rabbit. At least, the conclusion that cytolysins must have been formed in the chickens treated with the rabbit lenses because of the readiness with which this species produces cytolytic sera, is not tenable.

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INVESTIGATIONS ON THE CONTROL OF HOOKWORM DISEASE. XI. VERTICAL MIGRATION OF INFECTIVE HOOKWORM LARVAE IN THE SOIL (PRELIMINARY REPORT).*

By FLORENCE KING PAYNE, M.D.

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Plates XI to XIII.

INTRODUCTION.

There have been a number of studies of the migration of hookworm larvae on the surface of soil and on the walls of mine shafts, but investigations of their travels beneath the surface have been hampered by the lack of adequate means of isolating them from surrounding media. The development of a successful method of isolation (Cort, Augustine, Ackert and Payne, 1922; Baermann, 1917) has given an opportunity for the pursuit of studies of such migrations.

The vertical migrations of hookworm larvae may affect practical hookworm control in two chief ways: First, by the possible retirement of the larvae during dry weather from the surface to zones of more moisture. Second, by the possible migration upward of larvae which are beneath the surface. Studies of such migrations should assist in the evaluation of methods of excrement disposal, especially where burial is practiced or pit latrines are used. The present study considered only the second phase of vertical migration. Each experiment was based on the assumption that excrement containing fully developed larvae had been buried at a known distance beneath the surface. The object was to determine whether larvae were likely to reach the surface in sufficient numbers to produce an infective focus.

METHODS.

Samples of the three types of soil which were used were obtained in quantities of fifty pounds to one hundred pounds. Each was thoroughly mixed to obtain uniform distribution of the various constituents. Portions of each sample were dried at a temperature of about 60° C. and were pulverized. It was determined by experiment that drying at this temperature was sufficient to kill any nematodes or larvae, but was not high enough to char organic matter. The dried soil was kept as the stock from which material for the various experiments was obtained.

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* This paper is the eleventh of the series of hookworm investigations made by the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University, with the cooperation of the International Health Board, Rockefeller Foundation.

Containers for the experiments were of various sizes. The ones most used were tin sleeves, about three inches in diameter and five inches high. They were slightly tapered so that two or more could be joined to obtain additional length. Other containers of various sizes and forms were used in a few experiments and will be described with them.

Except where otherwise specified, only well developed *Necator americanus* larvae were used. The methods of culture and of isolation of the larvae have been given in an earlier paper of this series (Cort et al., 1922). In nearly all experiments the larvae were counted and were placed in a thin layer of moist soil on the bottom of the container.

The determination of the extent of upward migration accomplished by the larvae was made by removing the soil from the container in layers of measured thickness. Each layer was placed in an isolation apparatus and the larvae were recovered from it according to the method to which reference has been made. The larvae recovered from each layer were counted.

EXPERIMENTAL WORK.

Experiments were conducted to test the effect of the following factors on the upward migration of hookworm larvae:

1. Moisture content.
2. Conditions simulating the rise of ground water.
3. Type of soil.

Another series of experiments was made to study the disappearance of nutritive material from the bodies of the larvae at various stages of migration.

The first experiments were designed to test the effect on vertical migration of different degrees of moisture, uniformly distributed through the soil. In all of these experiments soil from the same lot

TABLE 1.

Influence of moisture content on vertical migration of hookworm larvae.
(The zones are measured from the bottom of the dirt in the containers, and represent the distances above the place of introduction of the larvae.)

Experiment.	Moisture in per cent.	Days.	Larvae inoculated.	Zone 1.	Larvae recovered from Zone 1.	Zone 2.	Larvae recovered from Zone 2.	Zone 3.	Larvae recovered from Zone 3.	Zone 4.	Larvae recovered from Zone 4.	Zone 5.	Larvae recovered from Zone 5.	Zone 6.	Larvae recovered from Zone 6.	Zone 7.	Larvae recovered from Zone 7.
1	41.4	4	507	0–¼″	51	¼″–1″	147	1″ –1¾″	9	1¾″–2½″	4	2½″–3″	0	....	....	....	....
2	41.4	9	611	0–¼″	30	¼″–1″	7	1″ –1¾″	2	1¾″–2½″	2	2½″–3¼″	0	3¼″–4″	0	....	....
3	40.0	6	455	0–½″	14	½″–1″	0	1″ –1½″	0	1½″–2″	0	2″ –2½″	0	2½″–3½″	0	....	....
4	42.2	4	4400	0–½″	962	½″–1″	83	1″ –1½″	25	1½″–2″	9	2″ –2½″	0	2½″–3″	0	3″–4″	0
5	40.6	13	995	0–½″	0	½″–1″	0	1″ –1½″	0	1½″–2″	0	2″ –2½″	0	2½″–3⅛″	0	....	....
6	42.7	7	4530	0–½″	345	½″–1″	51	1″ –1½″	1	1½″–2″	12	2″ –2½″	3	2½″–3″	4	3″–3½″	2
7	40.6	6	560	0–½″	91	½″–1″	17	1″ –1½″	0	1½″–2″	0	2″ –2⅜″	6	....	....	....	....
8	Water-logged	7	1137	0–½″	2	½″–1″	0	1″ –1½″	0	1½″–2″	0	2″ –2½″	0	2½″–3⅝″	0	....	....
9		3	347	0–¾″	2	¾″–1½″	2	1½″–2½″	1	2½″–3½″	0	3½″–4½″	0	....	....	....	....
10		2	1500	0–¾″	163	¾″–1½″	5	1½″–2¼″	0	2¼″–3″	0	3″ –3¾″	0	....	....	....	....

was used. It was typical of the soil in the region of Princes' Town and was made up of a mixture of marl, clay, and humus. For convenience it is designated in the description of the present experiments as "garden soil." The standard tin sleeve containers were used.

The experiments were performed during the rainy season and specimens of soil obtained from fields which were known to harbor hookworm larvae contained from 33.5 to 48.7 per cent. of water. In the laboratory, however, it was found impracticable to prepare a mixture of dried soil and water with uniform distribution, in which there was less than 40 per cent. of water. In the experiments with water-logged soil the earth was saturated throughout, and additional water was added so that the surface of the sample was always submerged.

Table 1 shows the results of a series of ten experiments which were made to test the influence of the moisture content on vertical migration of hookworm larvae. From specimens of which the moisture content was 40 to 42.7 per cent. (Table 1, Experiments 1–7), 1877 larvae were recovered. Of these, 1798 or 95.7 per cent. were in the lowest inch of the specimen; that is, in the periods of four to thirteen days between inoculation and recovery there was no evidence of effective migration. From the specimen left for nine days, only 6 per cent. of the larvae originally used were recovered, and from the specimen left for thirteen days no larvae were recovered.

From specimens which were water-logged (Table 1, Experiments 8–10), 175 larvae were recovered. Of these, only 8 had migrated more than three fourths of an inch. The supernatant water on these specimens was examined daily for larvae, and none was found in any experiment. It would appear that the death rate in these specimens was high.

The failure of extensive vertical migration to occur under conditions of relatively uniform distribution of moisture led to the attempt to simulate some conditions existing in nature. Various efforts were made to bring about experimental conditions which would resemble those existing in the soil during the rise of ground water from the water table to the drying surface.

In experiment No. 11 (see Table 2), dry soil was placed in a glass tube three eighths of an inch in diameter to a depth of three and one half inches. More than one thousand larvae were placed in the lower end on a little moist earth. This end was closed with a stopper into which was inserted a very slender glass tube bent in the form of a U.

The tube of earth was then placed in a vertical position. Water was introduced, a few drops at a time, through the U tube. Care was taken that the level of the water in the U tube should not be higher than the lower end of the column of earth in the larger tube. In the course of a few hours water had permeated throughout the soil. Of 345 larvae which were found in the top one fourth of an inch of the earth on the following day, 117 were recovered from the uppermost lump of earth which measured about one eighth of an inch in diameter.

In experiment No. 12 (see Table 2), one of the standard tin sleeves was used. This was set on a flat tin surface, and the larvae were placed in the bottom in a bit of moist earth. Dry earth was then poured into the sleeve to a depth of four inches, and in the center additional earth was piled to form a cone one half inch in height. A little water was placed on the flat surface outside the container, and it was renewed as rapidly as it was taken up by the soil. The surface of the soil in this container was examined from day to day. After twenty-four hours it was estimated that about 300 larvae could be found with a binocular microscope. The container was kept open and no attempt was made to prevent drying of the surface. After a few days only empty sheaths were visible on the surface of the higher lumps of earth, while larvae could be seen at lower levels. A control experiment was conducted in the same manner, but without larvae, and on the eighth day the moisture content of the soil was found to be 42.3 per cent.

In order to eliminate the possibility of larvae ascending the walls of the container instead of migrating through the earth, experiment No. 13 (see Table 2) was performed, with a container five inches square. This experiment was carried out in the same manner as No. 12, but drying for such a long time was not permitted.

In experiment No. 14 (see Table 2) there was used a deeper layer of earth than in the previous experiments, and in order to obtain a continuous water column from the bottom to the top, it was necessary to moisten the top layers of earth.

In the four experiments recorded in Table 2 migration was more successful. Of 1094 larvae recovered, only 165 or 15.1 per cent. were found within one inch of the bottom of the container. 814 or 74.4 per cent. had migrated three inches or more before recovery; 235 had migrated four inches or more, and 169 five inches or more. The greatest distance noted was in the case of three larvae, in experiment No. 14,

TABLE 2.

Influence of rise of ground water on vertical migration of hookworm larvae.

Experiment.	Days.	Larvae inoculated.	Zone 1.	Larvae recov'd from Zone 1.	Zone 2.	Larvae recovered from Zone 2.	Zone 3.	Larvae recovered from Zone 3.	Zone 4.	Larvae recovered from Zone 4.	Zone 5.	Larvae recovered from Zone 5.	Zone 6.	Larvae recovered from Zone 6.	Zone 7.	Larvae recovered from Zone 7.	Zone 8.	Larvae recov'd from Zone 8.
11	1	1000	$0-\frac{1}{4}''$	64	$\frac{1}{4}''-1''$	101	$1''-1\frac{3}{4}''$	36	$1\frac{3}{4}''-2\frac{1}{2}''$	39	$2\frac{1}{2}''-3\frac{1}{4}''$	8	$3\frac{1}{4}''-3\frac{1}{2}''$	345	....	....	....	...
12	8	600	$0-1''$	0	$1''-2''$	1	$2''-3''$	12	$3''-3\frac{1}{2}''$	53	$3\frac{1}{2}''-4''$	172	$4''-4\frac{1}{2}''$	32	....	....	....	...
13	4	?	$0-1''$	0	$1''-2''$	2	$2''-3''$	5	$3''-4''$	6	$4''-5''$	37	$5''-5\frac{1}{2}''$	105	$5\frac{1}{2}''-6\frac{1}{4}''$	46	....	...
14	10	1530	$0-1\frac{1}{2}''$	0	$1\frac{1}{2}''-2\frac{1}{2}''$	1	$2\frac{1}{2}''-3\frac{1}{2}''$	1	$3\frac{1}{2}''-4\frac{1}{2}''$	3	$4\frac{1}{2}''-5\frac{1}{2}''$	10	$5\frac{1}{2}''-6\frac{1}{2}''$	11	$6\frac{1}{2}''-7\frac{1}{2}''$	1	$7\frac{1}{2}''-8\frac{1}{2}''$	3

which were recovered from zone seven and one half to eight and one half inches from the bottom.

The experiments which have been described involved studies of migration in only one type of soil, and this a type which is common in relatively restricted areas. In order to determine whether migration might be more successful in other soils a preliminary series of experiments was made with two others which were available at the time. One of these was composed almost entirely of very fine white sand, the other was of sand mixed with finely divided particles of red clay. The experiments with soils were conducted in small containers with a depth of soil of about two inches. The technique was identical with that described in the experiments above which simulated the rise of ground water, except that the larvae were not recovered. The success of migration was tested by frequent search for larvae on the surface of the soil by means of a hand lens or binocular. Each experiment was controlled by a similar preparation in which garden soil was used.

Six series of experiments were made in the attempt to establish conditions under which successful migration would occur in these soils. The experiments with the white sand were uniformly unsuccessful. No larvae were found on the surface of this soil. In the case of the mixture of sand and red clay, there were found at the end of twenty-four hours in each experiment a few larvae, but never so many as were seen on the garden soil control.

The results of the experiments which have been described indicate that in the types of soil which have been studied vertical migration takes place through only a limited range, except under the most favorable conditions. Studies of the larvae, used in the experiments, showed that there was little or no migration, if the food granules were sparse, and anatomical details were readily made out. Larvae, which in the beginning were so thickly studded with granules that very little internal structure could be distinguished, showed, after migration of a few inches, a great diminution in granules and a general appearance similar to that pictured by Looss (1911) in a larva reared in a medium with insufficient nutriment. These observations led to a series of experiments to study the diminution in the number of granules in relation to the various distances migrated.

Six different lots of well developed and nourished larvae were used for this experiment, and with each lot several preparations of different depths were made according to the technique described for the experiments simulating the rise of ground water. The distribution of food granules was studied in the larvae of each lot, in the larvae which came to the surface of the preparations, and in the larvae which were recovered from various zones of the deeper preparations. Photomicrographs were made of typical larvae of each lot and of larvae from each stage of migration. The preparation of the photomicrographs was done by Dr. Geo. C. Payne, and acknowledgment is made to him for his assistance as well as to Dr. K. S. Wise, Surgeon-General, and Dr. Baeza, Acting Government Bacteriologist, for the use of necessary apparatus.

The experiments were conducted under fairly uniform temperature conditions. Room temperature varied from about 72 to 87° F. All experiments were carried through in as short a time as was consistent with the height of migration which was desired. Even the highest migration took place within less than a week from the preparation of the lot, and four days from the actual inoculation of the soil. Portions of the supply of stock larvae were retained in each instance and were kept in clean water as controls to show the changes in the distribution of food granules in inactive larvae. Observations on the controls showed that there was no appreciable diminution in the number of food granules during the period of any experiment. In interpreting these experiments it is to be understood that the total distance travelled by a larva is much greater than the vertical distance of apparent migration. Hand lens studies of the activities of larvae in vertical glass tubes containing earth showed that a larva frequently traverses the same zone many times before finally leaving it for a higher one.

Diminution in the number of food granules was shown after migration of one inch, which was the smallest distance at which studies were made, and when only eighteen hours had elapsed from the time of inoculation. The loss of granules was apparently progressive and constant with farther migration and with the passage of more time, so long as activity was maintained. Because of the devious courses taken by the larvae in their travels there was a certain amount of variation in the diminution of granules in larvae from the same lot and the same zone, but when representative individuals were selected from each zone the disappearance of the granules could be followed with a fair degree of accuracy. There appeared to be an early diminution in the number of granules in the anterior portion of the larvae, and this was followed by an apparent increase in the width of the lumen of the intestine, probably due to a decrease in depth of the intestinal cells. The accompanying figures show a characteristic series of such changes in larvae from one lot, and from different zones of migration up to six and three fourths inches (See Plates XI to XIII, Figs. 1–11).

SUMMARY.

1. In garden soil with uniform conditions of moisture throughout the sample very few *N. americanus* larvae migrated more than two inches above the point of inoculation.

2. In water-logged garden soil there was apparently a very high death rate and there was little migration.

3. In garden soil in which the conditions of rising ground water were simulated 74.4 per cent. of the larvae recovered had migrated three inches or more, 15.4 per cent. had migrated five inches or more, while a few had migrated more than seven and one half inches.

4. Migration was apparently more difficult in the specimens of fine sand and the mixture of clay and sand than in the specimens of garden soil.

5. The food supply carried by *N. americanus* larvae apparently restricts the range of their vertical migration to narrow limits, since a distinct reduction in the granules in the intestinal wall could be noted after vertical migrations of only an inch or two, and almost a complete loss of these granules came about in those which had migrated for five or six inches.

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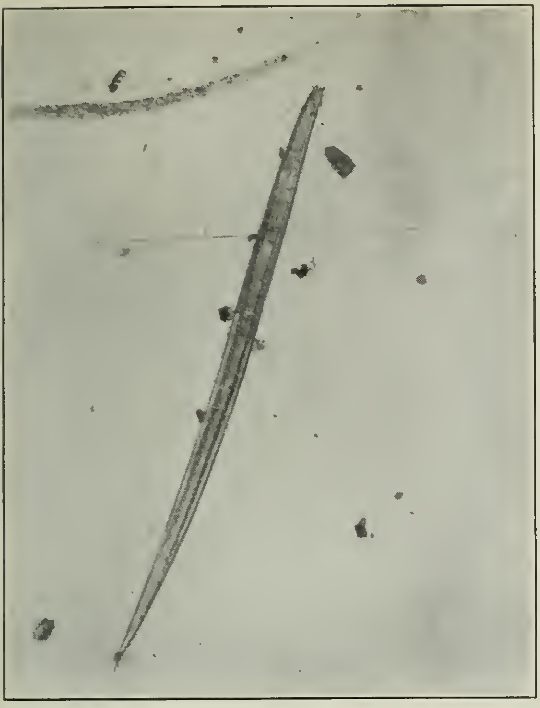

FIG. 1.

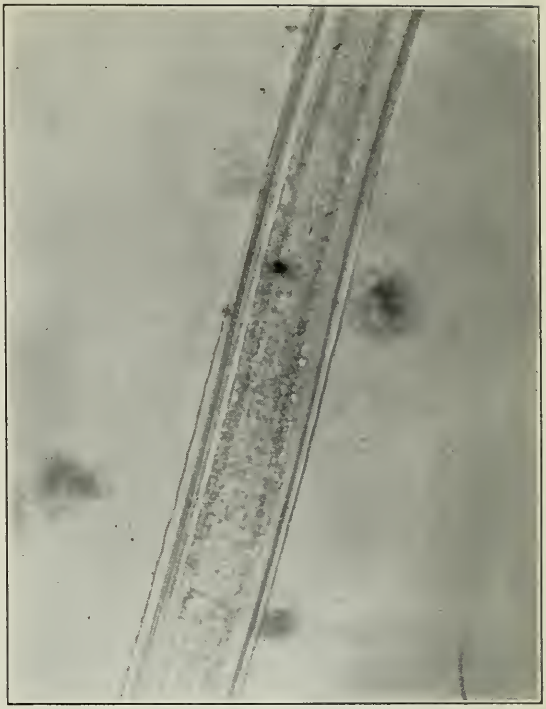

FIG. 2.

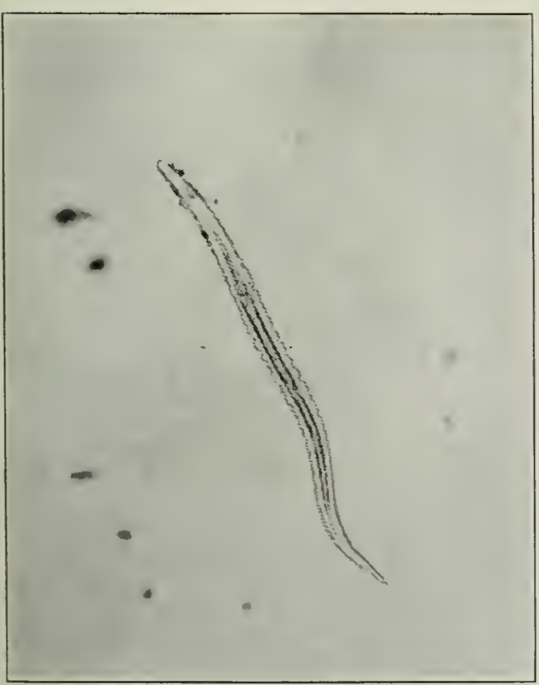

FIG. 3.

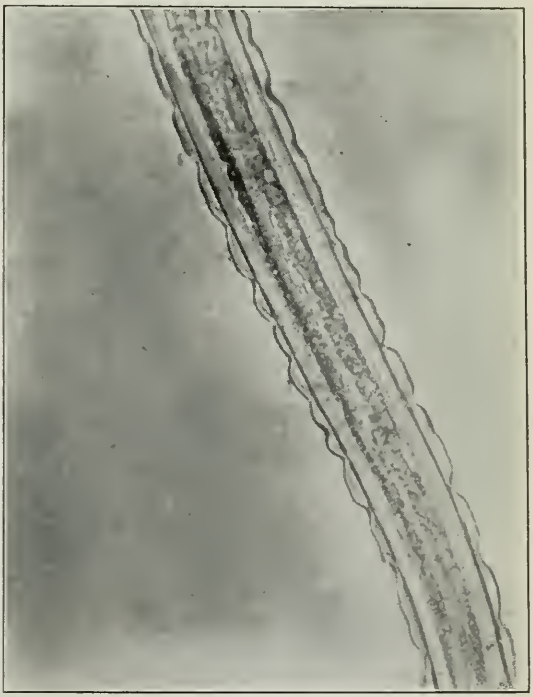

FIG. 4.

PLATE XII.

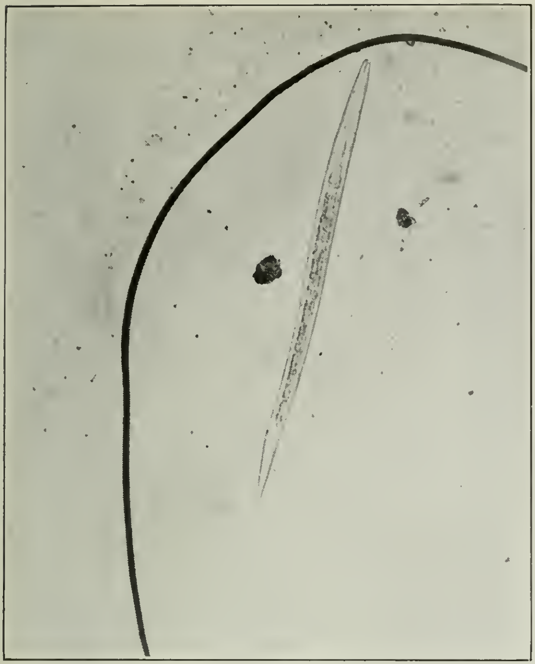

Fig. 5.

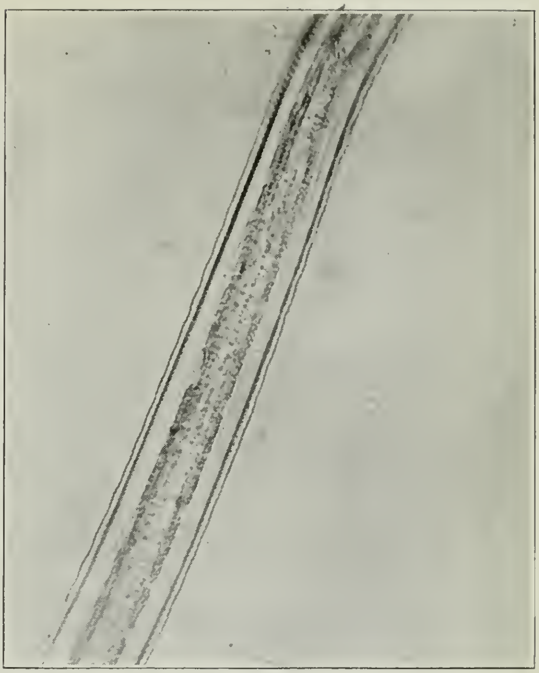

Fig. 6.

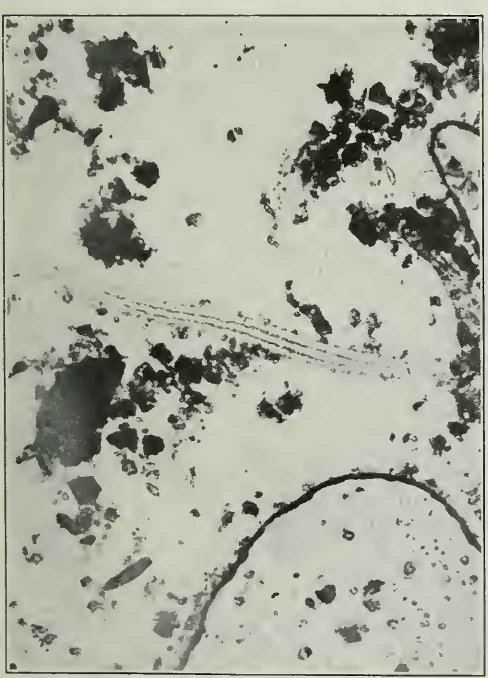

Fig. 7.

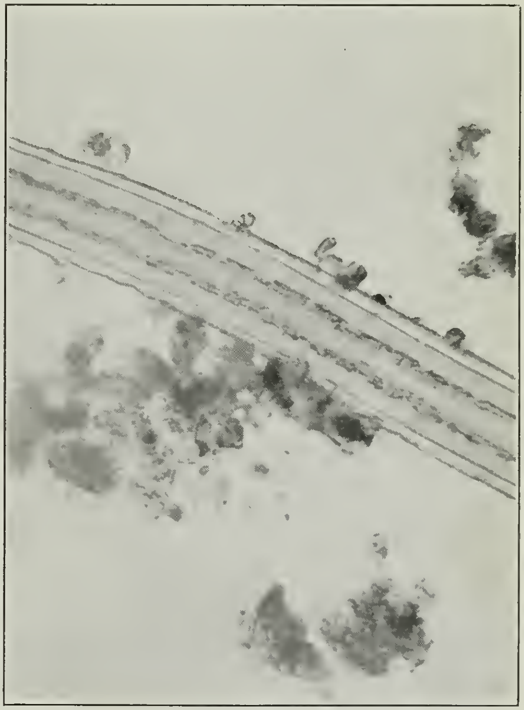

Fig. 8.

PLATE XIII.

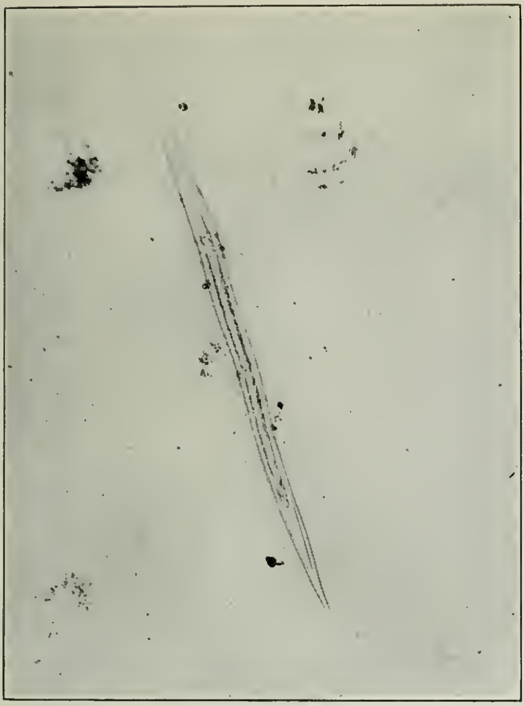

FIG. 9.

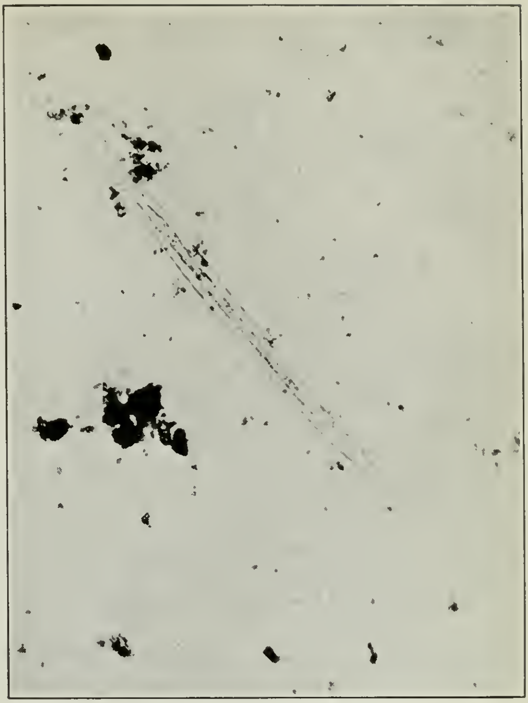

FIG. 10.

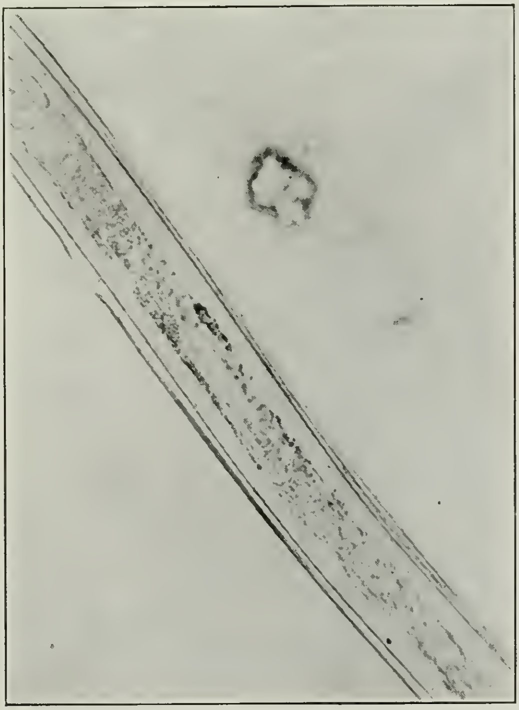

FIG. 11.

EXPLANATION OF PLATES.

Plate XI.

Fig. 1. Larva from lot No. 4, before migration, low-power.

Fig. 2. Larva from lot No. 4, before migration, high-power.

Fig. 3. Larva from lot No. 4, after migrating a vertical distance of two inches, low-power.

Fig. 4. Larva from lot No. 4, after migrating a vertical distance of two inches, high-power.

Plate XII.

Fig. 5. Larva from lot No. 4, after migrating a vertical distance of three and one half inches, low-power.

Fig. 6. Larva from lot No. 4, after migrating a vertical distance of three and one half inches, high-power.

Fig. 7. Larva from lot No. 4, after migrating a vertical distance of four and one half inches, low-power.

Fig. 8. Larva from lot No. 4, after migrating a vertical distance of four and one half inches, high-power.

Plate XIII.

Fig. 9. Larva from lot No. 4, after migrating a vertical distance of six and one half inches, low-power.

Fig. 10. Larva from lot No. 4, after migrating a vertical distance of six and three quarters inches, low-power.

Fig. 11. Larva from lot No. 4, after migrating a vertical distance of six and three quarters inches, high-power.

THE RESISTANCE OF DIFFERENT HOSTS TO EXPERIMENTAL TRYPANOSOME INFECTIONS, WITH ESPECIAL REFERENCE TO A NEW METHOD OF MEASURING THIS RESISTANCE.*

By W. H. TALIAFERRO, Ph.D., and L. G. TALIAFERRO, A.B.

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* From the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Md.

This is the second of a series of papers dealing with trypanosomes in which we are trying to attack some of the problems dealing with (1) variation and inheritance, and (2) the physiological factors involved in the interaction of host and parasite. The first paper of the series deals primarily with the first subject and is now in press for the Journal of Experimental Zoology (Taliaferro, 1922).

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INTRODUCTION.

It has long been known that the course of experimental trypanosomiasis varies with different species of trypanosomes and with different species of hosts. Or, in other words, the life history of a trypanosome in a mammal may vary with the species of trypanosome, or it may vary when the same trypanosome is grown in different vertebrate hosts. These different types of life-histories can be considered under four headings: In the first place, we find that in certain non-pathogenic trypanosomes (*T. lewisi* in the rat) the organisms, after a short incubation period, appear in the blood and undergo continuous and active multiplication. During this time they steadily increase in numbers in the blood. After from 22 to 32 days from the beginning of the infection they cease all reproduction and exist in the blood, sometimes in enormous numbers, without showing any reproductive activity. After a further interval of from 7 to 100 days, these so-called "adult" forms disappear from the blood and the rat is immune to a second infection with *T. lewisi.* In the second place, we find that many of the pathogenic African species (*T. brucei, rhodesiense,* etc.), when grown in rats and mice, exhibit a continuous type of infection. After an incubation period of several days, the organisms appear in the blood and continue to reproduce and multiply until the host dies. In the third place, the same pathogenic species, when grown in guinea pigs, dogs, etc., exhibit a peculiar relapsing type of infection, which is characterized by alternate periods, during which the trypanosomes in the blood increase or decrease in numbers. Finally, at least one species of trypanosome is known (*T. cruzi*) which apparently does not reproduce in the blood at all, but reproduces in the tissues,—a few of the resulting forms being liberated and living in the blood.

The chief differences between these four types of infection can be explained by assuming a peculiar type of constitution either in the given species of trypanosome or in the host. The peculiar life history of *T. cruzi* can probably be best explained on the first assumption. This is a tissue—rather than a blood-parasite. In the other cases, on the other hand, the chief underlying cause, which determines whether a given infection is of a continuous or a relapsing type, seems to be best explained by assuming that in certain hosts there is some condition (temporary or permanent) which is adverse to the trypanosomes, while in others there is little or no hindrance to the life and reproduction of the parasites. That this is the true explanation of these differences is supported by the work of Pearce and Brown (1918), who have shown that the same species of pathogenic trypanosome exhibits different, but characteristic life histories in different hosts. Even the peculiar type of infection shown by *T. lewisi* in the rat is modified into, what appears to be, a continuous type of lethal infection when this organism is grown in mice (Roudsky, 1911).

In the present work we shall consider any of these conditions which are adverse to the life of the parasite as *resistance*. Resistance in this sense may hence include both a direct resistance, the result of a specific activity on the part of the host, as well as an indirect resistance, resulting from the exhaustion of some essential foodstuffs.

With the exception of *T. cruzi*, with which we will not deal in this paper, a consideration of the facts given above seems to indicate that the highest grade of resistance is encountered in the case of a nonpathogenic trypanosome, such as *T. lewisi* in the rat. Here all reproduction of the parasites ceases 22 to 32 days after the beginning of the infection. The parasites then exist in the blood for 7 to 100 days and finally disappear altogether. Likewise the animal is immune to another infection with *T. lewisi*. The next grade of resistance is that which causes temporary decreases or even disappearances of the parasites from the peripheral blood. This type of resistance is observable in the relapsing infections, such as *T. brucei* in the guinea pig. Here the resistance is often very marked, but it is rarely ever complete, as the host almost invariably succumbs to the infection. In the continuous types of infection, such as *T. brucei* in the rat, there is very little, if any, resistance encountered by the parasites. Apparently the organisms, once they appear in the blood, continue to multiply until the host succumbs.

Any resistance to a trypanosome infection may do one or both of two things: (1) it may retard the rate of reproduction of the parasites, or (2) it may destroy them after they are formed. A fairly accurate picture of the sum total of the resistance of the host may be obtained by making daily determinations of the number of trypanosomes per cmm. of blood. If, for example, the parasites either remain constant or decrease in number for a certain period, we know some type of resistance is operative, but we do not know whether it is producing its effect by retarding the rate of reproduction of the parasites or destroying them after they are formed. That this is true is shown by a consideration of the following equation which gives the factors determining the number of parasites per cmm. of blood.

Number of parasites per cmm. of blood = Number produced by reproduction of parasites — Number destroyed, in consequence of the resistance.*

A further consideration of this equation shows that if we can supplement the enumerative studies with a measure of either the rate of reproduction of the parasites or with a measure of the rate of their destruction, we can differentiate between the two types of the resistance.

In the present work we have attempted to differentiate between the two different types of resistance by supplementing the daily counts with a measure of the rate of reproduction of the trypanosomes. It would probably be impossible to get an absolute measure of this rate unless we could preserve all of the progeny which are produced, and this is obviously not possible, if some are being destroyed. Our measure of this rate is, therefore, a comparative one, and is based on the difference in the variability in size in the individuals of a sample taken, on the one hand, from a population of organisms undergoing rapid multiplication, and, on the other hand, from a population of organisms undergoing little or no multiplication. It is a matter of common observation that a sample from a population of the first type, which necessarily consists of adults and many young forms in different stages of growth, will exhibit a higher variability in size than a sample from one of the second type, which consists largely of adult forms which are more or less uniform in size. Furthermore, it is very important to note that the greater the rate of reproduction with its production of young forms, the greater the variability in the size of individuals from samples of that population. In other words, it follows from these rather self-evident facts that a measure of the variability of size in the individuals of different samples will give us a comparative measure of the rate of reproduction (or production of young forms) in the different populations from which these samples are taken. Although such a method is an indirect one, its great advantage lies in the fact that it is independent of the number of individuals that are killed off or removed, as long as enough individuals are left to enable us to get a sample of suitable size for statistical analysis, and as long as the destructive agent does not select individuals of a given age.

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* The objection might be raised that a disappearance of the trypanosomes from the blood does not necessarily mean that the organisms are destroyed, because they may have simply migrated into the tissues. While trypanosomes undoubtedly do occur in the tissues, there is no evidence that they occur in greater quantities there, when they begin to decrease in the blood. On the other hand, Levaditi and McIntosh (1910) have found trypanolytic antibodies, produced after the crises in the relapsing types of infection, which indicates strongly that the trypanosomes are actually destroyed.

There are two methods of measuring the amount of variability in size of the individuals in a given sample. The first method is that of obtaining the standard deviation, which is a measure of the amount the individuals deviate from the mean or average, expressed in concrete units, such as microns. The second method is that of obtaining the coefficient of variation, which measures the deviation in percentages of the mean. The second constant is the more valuable from our standpoint, because it is a relative measure and allows us to compare the variability of two samples regardless of differences in their mean size.

The method of measuring the rate of reproduction by ascertaining the coefficient of variation in size of random samples is especially applicable in the case of the trypanosomes. In our work we have used the coefficient of variation for total length. The parasites reproduce almost exclusively by binary fission. During this process the organisms grow somewhat longer in size and then divide by an oblique longitudinal fission, producing one fairly long and one very short form. It is therefore evident that the higher the rate of reproduction the greater the number of short forms that will be produced, and in consequence the higher will be the coefficient of variation for total length.

Just how great a difference in variability is found between a sample taken from a population in which rapid reproduction is taking place and one in which there is little or no reproduction can be easily visualized by examining Fig. 1. This figure shows the lengths of two sets of *T. lewisi* from rat 105. Set *A* contains ten trypanosomes, drawn at random, and arranged according to the order of their decreasing lengths, from a slide made the first day after trypanosomes appeared

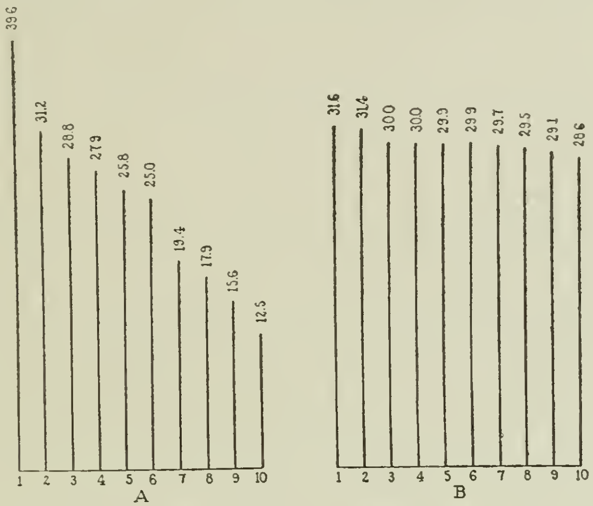

FIG. 1. The lengths of two samples of trypanosomes from rat 105 plotted according to scale and arranged in order of their decreasing lengths. The actual length in microns is also shown. *A*, ten specimens taken at random from the fourth day of the infection; *B*, ten specimens taken at random from the twenty-eighth day. Note the great variability in length of those specimens (*A*) known to be undergoing rapid reproduction, and the uniformity in length of those (*B*) which are known not to be reproducing.

in the blood, at which time they are known to be multiplying actively. Set *B* is a similar set of ten trypanosomes drawn from a slide made on the twenty-eighth day of the infection, at which time all multiplication is known to have ceased. The great variability in size in those specimens (set *A*) which are undergoing rapid multiplication, and those (set *B*) which are not, is evident. While the coefficient of variation was not worked out for these actual samples, it was compiled for larger samples (100 individuals), taken at the same time. The coefficient of variation for a sample of 100 individuals from the same slides from which set *A* was drawn was 25.32 per cent. and that for a similar sample from the slides from which set *B* was drawn was 2.80 per cent.

The rationale of the use of the coefficient of variation for total length as a measure of the rate of reproduction is, we believe, evident from this discussion. Since the conclusions reached in the following experiments rest on the assumption that this is a valid method, we feel that it is necessary to completely justify its use. It has to be shown, for example, that the method actually works for trypanosomes, and that no errors are introduced by the possibility mentioned above, namely, the selective killing of trypanosomes of a given age and size, but so much of the proof of the validity of the method rests on the data given in the following experiments that we will present these first. We will, therefore, tentatively assume that the method is valid, and will defer a more complete discussion of it until after the data have been presented.

A. The problem. A brief recapitulation of the problems and methods of attack which are dealt with in this paper may be given as follows: The resistance which a host offers toward a trypanosome infection may either retard the rate of reproduction of the parasites or may actually destroy them after they are formed. These two types of resistance can be differentiated: (1) by making daily counts of the number of trypanosomes per cmm. of blood, and (2) by ascertaining the comparative rate of reproduction of the trypanosomes as is indicated by their coefficient of variation for size. These determinations make it possible to ascertain the relative value of the two types of resistance, because the number of parasites per cmm. of blood at a given time = the number produced by the reproduction of the parasites — the number destroyed in consequence of the resistance, and, if we can ascertain any two of these factors, the third can be estimated. The validity of this method of attack has already been discussed, and will be taken up in greater detail on page 305.

B. Material. The different strains of trypanosomes used in this work have been obtained from several sources. Our strain of *T. lewisi* was obtained from wild Norway rats living on one of the Baltimore city dumps. *T. equiperdum* was furnished to us by Dr. John S. Buckley of the Bureau of Animal Industry. Our *T. equinum* is from Dr. F. G. Novy's strain, and was secured through the kindness of Dr. Paul A. Lewis of the University of Pennsylvania. Both *T. brucei* and *T. rhodesiense* were received from Dr. Wade H. Brown of the Rockefeller Institute. Dr. Brown informs us that there is some uncertainty as to whether the strain which we have designated *T. rhodesiense* is in reality this species. According to him the strain came originally from a patient in East Africa supposedly infected with *T. rhodesiense.* After it was kept at the Rockefeller Institute for upwards of a year it showed certain morphological changes which have raised some question in regard to its identity. From the standpoint of the present work, however, this is not of very great importance. It is a pleasure to acknowledge our great indebtedness to these investigators, without whose courtesy we would have been unable to carry out the present work. Mention should also be made of the valuable assistance received from our laboratory assistant, Mr. Conrad Bauer.

C. Measurements of size. As our measure of the rate of reproduction of the trypanosomes is based on determinations of the coefficient of variation in size, of samples taken from different infections, it is necessary to select some distance on the body of the trypanosomes, as a measure of size, whose magnitude is different in young and adult forms, and whose determination can be easily and accurately made. A consideration of the structure of a trypanosome suggests several such measurements. The shape of the body in *T. lewisi*, for example, can be seen by an examination of Fig. 2. The chief organelles are a more or less centrally placed nucleus, a blepharoplast from which a flagellum arises, and a parabasal body which is generally so closely associated with the blepharoplast as to obscure it. The proximal portion of the flagellum which extends along the side of the body is united with the latter by the so-called undulating membrane. In the pathogenic species these structures are slightly modified in that the total length of the body is less, the width is greater, the distance from the posterior end to the parabasal body is less, and the parabasal body is smaller.

In our work on *T. lewisi* and *T. equiperdum*, we calculated the coefficient of variation for all of the six distances shown in Fig. 2, viz., (1) posterior end *to* parabasal body, (2) parabasal body *to* nucleus, (3) nucleus *to* anterior end, (4) anterior end *to* end of flagellum, (5) total length, which includes these four measurements, and (6) width, which was taken midway between the parabasal body and the nucleus. It can readily be seen that all of these distances are smaller in specimens which have just divided (young forms) than in specimens just before division (" adult " forms). It was found, therefore, that a study of the coefficient of variation of any of these distances indicated the changes in the rate of reproduction. After a careful consideration of the various measurements, however, we decided to use only total length. This distance is undoubtedly the best single measurement of size for our purpose because: (1) it is the longest distance, and hence, personal errors in measuring it are proportionately less than for the shorter distances, and (2) some of the distances between organelles are intrinsically more variable due to the shifting back and forth of the organelles (see Taliaferro, 1922).

D. Computations. As we have pointed out above, we can measure the degree in variation of size in samples by obtaining either the standard deviation or the coefficient of variation. The latter constant is the more valuable from our standpoint, because it expresses the standard deviation as a percentage of the mean, and is, therefore, available in comparing the degree of variation between samples possessing different mean values. It can be expressed by the following formula:

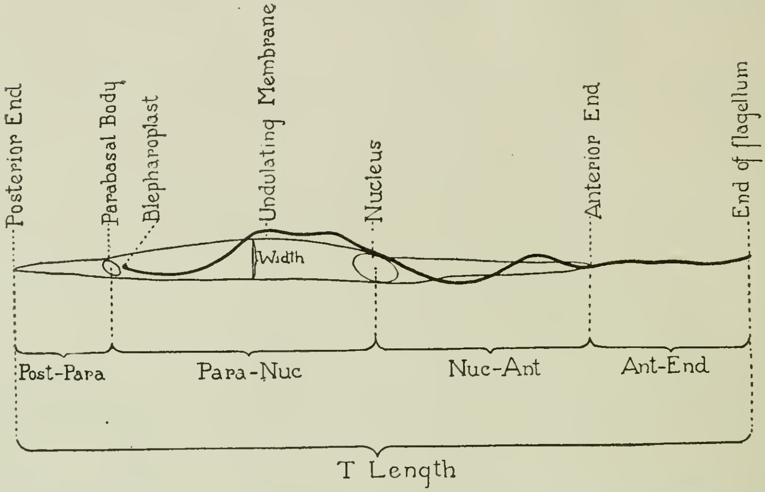

FIG. 2. Diagram of *Trypanosoma lewisi,* projected along a straight line, showing the parts of the organism and the distances used in determining variability in size. *Total length* was found to be the most suitable of these distances, and is the one used in this study. The designation of the parts of the body run vertically, while those of the distances run horizontally.

$$\text{C. V.} = \frac{100\sigma}{M}.$$

in which σ is the standard deviation and M the mean. The mean is the average value, and is obtained by dividing the sum of the various measurements by the number of individuals ($\Sigma x/n$). In obtaining the standard deviation we have used the following formula:

$$\sigma = \sqrt{\frac{\Sigma x^2}{n} - M^2},$$

in which M is the mean, n is the number of individuals, and x denotes the magnitude of the measurements, *not their deviations.*

It is impossible to form any idea of the statistical validity of the constants obtained from these formulae unless the probable error is known. For this reason the probable error for most of the constants has been worked out with the aid of Pearson's Table for Statisticians and Biometricians. With the exception of the data for *T. lewisi*, it seemed unnecessary to give the probable errors for any more than the constants of one day of each infection, because they vary very little in magnitude from day to day, and are all computed from samples of the same number (i.e., 100 individuals). In the case of *T. lewisi* the probable errors are given for the first and last days of the infection. Besides these probable errors Table 1 is appended which gives the probable errors of the coefficient of variation for the integral numbers 2 to 19 per cent., where *n* equals 100. This table enables one to ascertain approximately the probable error of all of the coefficients of variation the probable errors of which are not given in the tables.

TABLE 1.

The probable errors (P. E.) for the coefficient of variation (C. V.) from 2 to 19 per cent. where n equals 100.

C. V.	P. E.	C. V.	P. E.	C. V.	P. E.
2.00	.09	8.00	.38	14.00	.68
3.00	.14	9.00	.43	15.00	.73
4.00	.19	10.00	.48	16.00	.78
5.00	.23	11.00	.53	17.00	.83
6.00	.28	12.00	.58	18.00	.88
7.00	.33	13.00	.63	19.00	.93

E. Enumeration of trypanosomes. Several methods have been devised for counting blood-inhabiting protozoa, such as trypanosomes. One of these methods is that of Kolmer (1915), who dilutes the blood with a fluid which hemolyzes the red cells, and then counts the trypanosomes with the ordinary blood counting methods. Ross and Thomson (1911), in their enumerative studies on trypanasomes, made a thick smear of a known quantity of blood, and then, after plasmolyzing the red cells, counted the trypanosomes on the slide. In our work we made red blood cell counts for our various experimental animals, and then made thin, even blood smears from them. After these blood smears were stained, the red blood cells and trypanosomes in a large number of microscopic fields were counted and the ratio between them calculated. Knowing the number of red cells per cmm. and the ratio of trypanosomes to the red cells, it was an easy matter to compute the number of trypanosomes per cmm. of blood. In a number of cases we checked our method with that of Kolmer and the two gave very closely comparable results.

F. Microscopical technique. In staining trypanosomes, preparatory to measuring them and computing their coefficient of variation for total length, it is necessary to select a technique which will distort the organisms as little as possible, because such distortion probaby would affect their variability in size. In a previous paper on size and variability in *T. lewisi* (Taliaferro, 1922) we came to the conclusion, in agreement with Minchin (1909), that the truest size relationships in this trypanosome can be obtained by fixing in osmic vapor, followed by one of the Romanowsky stains, and drying the slides in an early stage of the process. These dried specimens give the truest size relationships although they are not as suitable for cytological work as the ''never-dried'' or wet preparations. In the present study, this technique was followed in the case of *T. lewisi.* It is given in detail in the foregoing paper (Taliaferro, 1922). The technical difficulties of this process were so great, however, that for the pathogenic trypanosomes, studied in this paper, we used the ordinary clinical technique of making thin blood smears, drying them, without previous osmic acid vapor fixation, and staining in Giemsa's blood stain. We have already shown, in the above paper, that the omission of the osmic vapor fixation causes a slight shrinkage in size, but it does not produce an appreciable effect on the coefficient of variation, and hence, is perfectly well suited for our purposes, because we are not interested in size *per se*, but in variability in size.

After the slides were stained, they were examined and the midline of a hundred trypanosomes, from each, drawn at a magnification of 3,000, with the aid of a camera lucida. These midlines were then stepped off with a pair of dividers, the points of which were placed 3 mm. apart. The number of steps obtained for each line represented the length of the trypanosome in microns. It is to be emphasized that in drawing specimens from a given slide, every specimen was drawn just as it was encountered, until the requisite number was obtained. In other words, there was no selection of the individuals, but the 100 trypanosomes represented, as near as possible, a true random sample.

G. Experimental procedure. In presenting the various experimental trypanosomiases which follow, we have classified them according to the host rather than the species of trypanosome. This was thought advisable because, with a few exceptions, the species of host, rather than the species of trypanosome, seems to be the factor which determines the type of the resulting infection. Since our object in all these experiments was the same, viz., to differentiate that type of resistance which retards the rate of reproduction of the trypanosomes, and that type which destroys them after they are formed, the experimental procedure in all was the same. The host animal was inoculated intraperitoneally and its blood examined until trypanosomes appeared in the peripheral circulation. As soon as these could be demonstrated, blood smears were made at frequent intervals throughout the course of the infection and were stained, as indicated under *Microscopical Technique.* At the same time the slides were made, the red blood cells were generally counted with an ordinary hemocytometer. The slides were first examined and 100 trypanosomes drawn and measured. From these 100 measurements the coefficient of variation for total length for that day was calculated. The slides were then examined again and the ratio between red cells and trypanosomes ascertained, which, in conjunction with the red blood cell count, enabled us to estimate the number of trypanosomes per cmm. of blood.

THE RAT AS A HOST OF TRYPANOSOME INFECTIONS.

A. A non-pathogenic infection. We will consider a non-pathogenic trypanosome first, because the very fact that it is not pathogenic to its host indicates that the host builds up a high grade of resistance against it. Elsewhere (Taliaferro, 1921 *a* and 1922), we have described the infection with *T. lewisi* in the rat in great detail and it will suffice to merely give a summary of the facts here. The course of the infection can be divided as follows:*

1. Multiplicative stage (twenty-two to thirty-two days).
    - *a.* Incubation period during which no organisms are in the blood (four to eight days).
    - *b.* Multiplicative period in the blood (eighteen to twenty-four days).
2. Stage of "adult" infection (seven to one hundred days).

---

* In one of these papers (Taliaferro, 1921 *a*), we gave the different stages in the infection in terms of the *blood infection*, which starts only after the organisms appear in the blood. We now give these stages in terms of the entire infection which starts at the time of injection. This accounts for the apparent difference in terminology given here and in that paper.

During the multiplicative stage the organisms are undergoing more or less rapid reproduction, or at least growth, whereas during the "adult" stage of the infection they exist in the blood without any signs of division or growth. At the end of the "adult" stage, they disappear from the blood, and the rat is immune to another infection with this form.

T. lewisi: rat 105.

Protocol 105. Culture rat infected with *T. lewisi.* Injected intraperitoneally with 1 c.c. of a 1–100 dilution of blood from rat 116 on May 1, 1920.[*] Organisms first demonstrated in the blood on May 5, 1920. Trypanosomes disappeared from the blood on June 8, 1920, and were never found after this date, although repeated blood examinations were made. Trypanosomes were measured and the coefficient of variation for total length was calculated on 12 days, as is shown in Fig. 3 and Table 2. Estimations of the number of organisms per cmm. of blood were made on 13 days, as is shown in the same tables.

Incubation period was 4 days.

Duration of infection was 35 days.

TABLE 2.

Mean, standard deviation, and coefficient of variation for total length on 12 days, and the number of trypanosomes per cmm. of blood on 13 days during the course of the infection with T. lewisi in rat 105.

Each statistical constant is based on the measurement of 100 individuals, 1200 trypanosomes having been measured during this infection. See Fig. 3 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	4 days.	6 days.	8 days.	10 days.	12 days.	14 days.
Mean length	24.785±.423	27.668	30.108	30.519	....	29.904
Standard deviation	6.277±.299	5.226	4.159	1.206		1.453
Coefficient of variation	25.32±1.28	18.88	13.81	3.95		4.85
Number of trypanosomes per cmm. of blood	41,000	277,000	301,000	338,000	145,000	76,000

Interval after injection.	18 days	22 days	26 days	28 days	30 days	33 days	35 days.
Mean length	30.481	31.412	31.723	31.251	31.490	31.815	30.654±.069
Standard deviation	1.788	.977	1.250	.875	.952	.884	1.036±.049
Coefficient of variation	5.86	3.11	3.94	2.80	3.02	2.78	3.38±.16
Number of trypanosomes per cmm. of blood	67,000	69,000	62,000	57,000	56,000	52,000	45,000

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[*] All dilutions of blood were made with 0.9 per cent saline.

The appearance and increase of the organisms in the blood is directly dependent upon the rate of reproduction of the trypanosomes. For this reason let us first consider the changes in the rate of reproduction of the parasites throughout the course of the infection, and then take up the question of the changes in the number of organisms per cmm. of blood. If the changes, which we find occurring in the rate of reproduction of the parasites, explain the changes in the number per cmm., it will indicate that the resistance built up by the host is directed solely toward an effect on the rate of reproduction. If, however, the changes in this rate do not account for all the changes in numbers, it will be necessary to look for another type of resistance directed toward the parasites after they are formed. The changes in the coefficient of variation which, as we have already discussed, should give us a comparative measure of the rate of reproduction and the changes in the number of trypanosomes per cmm. can be seen by an examination of Table 2 and Fig. 3.

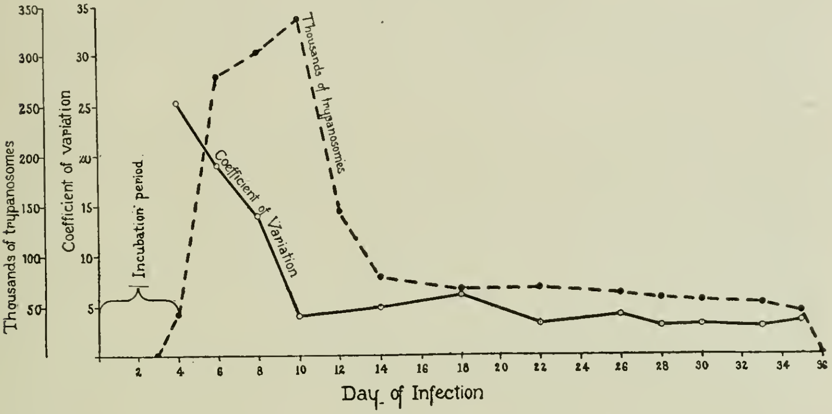

FIG. 3. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection with *T. lewisi* in rat 105.

Such an examination shows that the coefficient of variation for total length was unusually high (25.32 per cent.) on the fourth day of the infection, when the organisms were first demonstrated in the blood. Its value rapidly declined, so that by the tenth day of the infection it was only 3.95 per cent. There was then a very gradual decline until it reached the low value of 2.80 by the twenty-eighth day, from which value it did not show any appreciable change during the rest of the infection. We have already called attention to Fig. 1

which shows graphically the difference in variability between the organisms on the fourth and twenty-eighth days of this infection. We do not feel that there is any doubt that these changes are due to changes in the rate of reproduction of the trypanosomes. When they first appear in the blood, a microscopical examination of the slides shows that they are undergoing rapid multiplication. Actual division probably ceases by the tenth day. The slight decline noticed from the tenth to the twenty-eighth day is probably the result of the growth of a few young forms still in the blood. From the 28th day to the end of the infection, there is no morphological evidence either of division or growth.

The question might be raised, if the coefficient of variation gives a comparative measure of the rate of reproduction, how can we say there is no reproduction taking place when the coefficient of variation is between 3 and 4 per cent. This cannot be answered with absolute certainty, but we know that a population of adults undergoing no reproduction still exhibits a certain amount of variability in size, although the degree in variation is far less than for the same population undergoing rapid reproduction. These conclusions concerning the reproduction of the trypanosomes, which are drawn from a study of the changes in the coefficient of variation for total length, are supported by a microscopical study of the slides and are discussed in detail on page 306.

From the standpoint of the host's resistance to the infection, these results indicate that the host quickly built up a resistance which retarded the rate of reproduction more and more, so that all actual division of the parasites was inhibited by the tenth day of the infection. Let us see how these facts tally with the changes in the number of parasites per cmm. of blood. During the period extending from the first day after the trypanosomes appeared in the blood until the tenth day of the infection the parasites rapidly increased in numbers, reaching the high number of 338,000 per cmm. of blood. The rate of increase during this period, however, was not uniform, but was more rapid during the first two days than the last part of the period. In other words, the rate of increase was progressively less during this period, which is to be expected since our study of the coefficient of variation for total length indicated that the rate of reproduction was also progressively less during the same period. From the tenth to the fourteenth day, the parasites suddenly decreased in number (76,000 per cmm. on the fourteenth day). This sudden decrease can not be accounted for on the basis of the cessation of reproductive activity alone, because, had this been the only factor involved, the number of parasites would have remained stationary. We must, therefore, conclude that, at this point, the host has built up a resistance not only toward a retardation of the rate of reproduction, but also toward an actual destruction of the parasites after they are formed. From the fourteenth day until the thirty-fifth day, the number of parasites in the blood remained practically constant with the exception of a slight decrease. Between the thirty-fifth and thirty-sixth days they completely disappeared from the blood and could never be found again although repeated attempts were made to demonstrate their presence.

To sum up the results obtained in this infection with *T. lewisi* in the rat, we find: (1) A resistance was built up which retarded the rate of reproduction of the parasites more and more, and finally inhibited it altogether (by the tenth day). It is true that growth of the organisms may have taken place for some days more, but apparently all actual multiplication was stopped by this time. (2) Between the tenth and fourteenth days, a resistance was developed which killed off a large number of the parasites, i.e., it was directed toward their destruction after they were formed. Finally, (3) a resistance of the second type was again developed by the thirty-fifth day which caused the complete destruction of all the remaining organisms.

In the present paper we will limit our discussion simply to the different *effects* of the host's resistance and not its *nature*. It is possible that these different effects are due to the same fundamental type of resistance. This is notably true of the first and second effects given above. We will call attention to the fact, however, that the agency which finally destroys the trypanosomes at the end of the infection and that which causes the first decline in numbers is probably different. We feel this to be true, because the time which elapses between the two varies to such an extent. In the present infection this interval was about twenty-five days, but it may vary from a week to several months.

Rat 116.

Protocol 116. Culture rat infected with *T. lewisi.* Injected intraperitoneally with a single trypanosome, which was isolated with a Barber pipette, on April 8, 1920. Organisms first demonstrated in the blood on April 16, 1920. Trypanosomes disappeared from the blood on June 26, 1920, and were never found after this date, although repeated blood examinations were made. No clinical symptoms were observed during the course of the infection. Trypanosomes were measured and the coefficient of variation for total length calculated on 6 days, as is shown in Fig. 4 and Table 3. Estimations of the number of organisms per cmm. of blood were made on 5 days as is shown in the same figure and table.

Incubation period was 8 days.

Duration of infection was 79 days.

TABLE 3.

Mean, standard deviation, and coefficient of variation for total length, and the number of trypanosomes per cmm. of blood on 6 days during the infection with T. lewisi in rat 116.

Each statistical constant is based on the measurement of 100 individuals; 600 trypanosomes having been measured during this infection. See Fig. 4 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection	9 days.	25 days.	32 days.	39 days.	49 days.	79 days.
Mean length......	26.029±.470	29.481	30.551	29.271	31.629	31.162±.099
Standard deviation	6.978±.332	2.061	.982	.928	.850	1.469±.070
Coefficient of variation..........	**26.80±1.36**	**6.99**	**3.21**	**3.17**	**2.68**	**4.71±.22**
Number of trypanosomes per cmm. of blood........	....	**504,000**	**164,000**	**68,000**	**43,000**	**12,000**

Fewer determinations were made during this infection than in the previous one. An examination of Fig. 4 and Table 3 shows, however, that the results obtained support the conclusions reached in the pre-

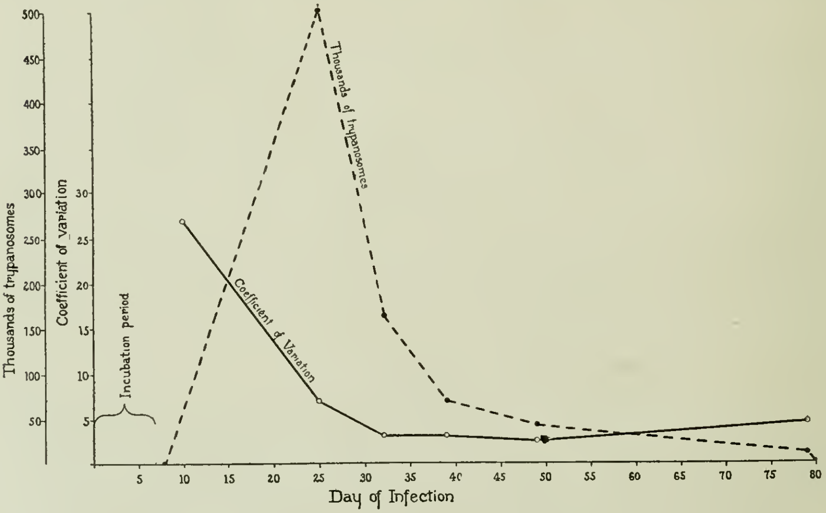

FIG. 4. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection with *T. lewisi* in rat 116.

vious experiment in a striking manner. The coefficient of variation for total length on the second day after the trypanosomes appeared in the blood (the ninth day after infection) was very high (26.80 per cent.), but it rapidly decreased, so that it was only 3.21 per cent. on the thirty-second day, from which value it probably did not change throughout the rest of the infection. It is true that there was a slight increase on the seventy-ninth day, but this change is so small in comparison with the changes noted at the beginning of the infection that it can probably be looked upon as an experimental error, rather than any actual change in the trypanosomes. These results indicate that the host has built up a resistance which is directed toward a retardation of the rate of reproduction of the parasites, so that by the thirty-second day of the infection all reproduction has ceased. This is similar to the results obtained for rat 105 except that a longer time elapsed before all reproduction was inhibited—a result probably connected with the fact that this infection was started with a single organism.

The changes in the number of trypanosomes per cmm. are also closely similar to those observed in rat 105. There was an initial rapid rise in the number of trypanosomes so that there were 504,000 on the twenty-fifth day of the infection. From the twenty-fifth day to the thirty-ninth day, they disappeared from the blood at a rate almost equal to their initial rise, so that by the latter day there were only 68,000 per cmm. From the thirty-ninth day to the seventy-ninth day the decline was very much more gradual. On the seventy-ninth day, however, there were only 12,000 per cmm. in the blood, and within the next twenty-four hours these totally disappeared, never to reappear. Just as in rat 105, then, there is a resistance built up which is directed toward a destruction of the parasites causing a marked decrease at the end of the multiplicative period and their total disappearance at the end of the infection.

Our general results in regard to the changes in the number of trypanosomes per cmm. of blood during the course of infections with *T. lewisi* are similar to those obtained by Steffan (1921).* This author, however, did not consider these changes from the standpoint of the present work.

B. Pathogenic infections of a continuous type. By far the majority of infections with pathogenic trypanosomes in the rat are of the continuous type. From the standpoint of the resistance built up by the host, these infections present just the opposite conditions to those found in a non-pathogenic trypanosome such as *T. lewisi.* In the following experiments we have studied *T. equiperdum* and *T. rhodesiense,* both of which show this type of infection in the rat.

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* Steffan also describes an acute type of infection with T. lewisi in which the parasites increase in the blood until the host dies. Recently we have found similar lethal infections with this trypanosome and are studying them with the methods given in the present paper.

T. equiperdum: rat 619.

Protocol 619. Culture rat infected with *T. equiperdum.* Injected intraperitoneally with 1 c.c. of a 1–100 dilution of blood from a guinea pig which had a heavy infection, on February 17, 1921, at 10:00 A.M. Organisms first demonstrated in the blood on February 19, 1921, at 9:00 A.M. Rat died on February 23, 1921, at 8:00 A.M., the last slides being made during terminal convulsions. Trypanosomes were measured and the coefficient of variation calculated for total length, and estimations of the number of organisms per cmm. of blood were made at 8 periods during the course of the infection, as is shown in Fig. 5 and Table 4.

Incubation period was 1 day 23 hours.

Duration of infection was 5 days 22 hours.

TABLE 4.

Mean, standard deviation, and coefficient of variation for total length, and the number of trypanosomes per cmm. of blood at 8 intervals during the course of the infection with T. equiperdum in rat 619.

Each statistical constant is based on the measurement of 100 individuals, 800 trypanosomes having been measured during this infection. See Fig. 5 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	2 days 10 hrs.	3 days.	3 days 10 hrs.	3 days 22 hrs.	4 days 12 hrs.	4 days 22 hrs.	5 days 11 hrs.	5 days 22 hrs.
Mean length	23.640±.117	23.812	22.448	24.552	24.880	24.792	23.976	23.574
Standard deviation	1.735±.082	1.788	1.821	1.687	2.280	2.562	2.191	2.579
Coefficient of variation	7.34 ±.35	7.51	8.14	6.87	9.16	10.33	9.13	10.94
Number of trypanosomes per cmm. of blood	7,000	38,000	229,000	194,000	1,333,000	1,710,000	3,692,000	5,102,000

Even a casual glance at Fig. 5 and Table 4, which give the results obtained for this infection, show that the changes both in the coefficient of variation and the number of parasites are entirely different from those of *T. lewisi*. The coefficient of variation was 7.34 per cent. on the first day after the trypanosomes appeared in the blood. This value is considerably lower than that for the same day in the *T. lewisi* infections. Instead of getting lower, however, as it did in the latter infections, it rose until at the time the animal died it was 10.94 per cent. There is no doubt that this increase in the coefficient of variation is significant from the statistical standpoint, because the difference between the constants in these two days is approximately 6 times its probable error. (10.94 ± .52) — (7.34 ± .35) = (3.60 ± .62). The question then arises is this great difference actually due to an increase in the rate of multiplication of the parasite or simply to some experimental error in technique? This question can not be answered until data for more infections of this kind are obtained. The general shape of the graph, showing the changes in the coefficient of variation, indicates that the rise is significant. We are at least justified in concluding that the host does not build up any resistance which is directed toward a retardation of the reproduction of the parasites, as was the case in the non-pathogenic infection with *T. lewisi*. There is, on the contrary, some evidence that the parasite gradually overcomes more and more the natural resistance which the host had at the beginning of the infection.

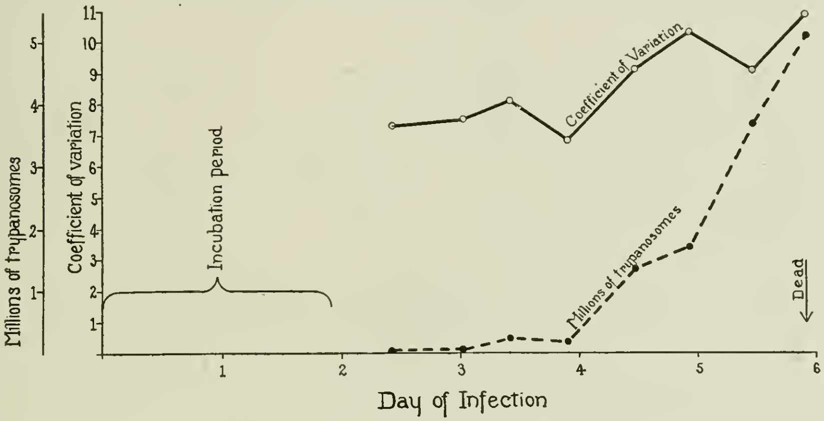

FIG. 5. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection with *T. equiperdum* in rat 619.

In an infection of this nature in which there is very little change in the rate of reproduction of the parasites from day to day, we would expect them to increase in the blood according to a curve of geometrical progression. An examination of Table 4 and Fig. 5 shows that there were 7,000 trypanosomes per cmm. of blood at the time of the first determination and that this number rapidly increased until there were 5,102,000 at the time of death. The curve of increase does not exactly follow a curve of geometrical progression. It does, however, strongly suggest a curve of this nature. In this connection it may be noted that the truest approximations to curves of the geometrical progression type were found in the continuous infections in the mouse. In spite of the fact that the trypanosomes did not increase in the blood exactly according to a curve of geometrical progression, we are justified in concluding that the host built up very little, if any, resistance directed toward a destruction of the parasites after they are formed. The results of the preceding paragraph also justify the conclusion that there was no resistance built up which retarded their rate of reproduction. On the contrary there is some evidence that they multiplied faster at the end than at the beginning of the infection.

T. rhodesiense: rat 703.

Protocol 703. Culture rat, infected with *T. rhodesiense.* Injected intraperitoneally with 0.5 c.c. of a 1–200 dilution of blood from a very heavily infected rat, on May 3, 1921. Organisms first demonstrated in the blood on May 4, 1921. Rat was dead by May 11, 1921. Calculations of the coefficient of variation for total length, based on the measurement of 100 individuals, and estimations of the number of organisms per cmm. of blood were made 4 times during the course of the infection, on the dates shown in Fig. 6 and Table 5.

Incubation period was 1 day.

Duration of the infection was approximately 6½ days.

The infection in this animal was very similar to the infection of *equiperdum* in rat 619. Both infections were of a very acute type and lasted approximately six days. While the coefficient of variation for total length is, on the average, higher than in rat 619, the changes are of approximately the same character. When the organisms first appeared in the blood the coefficient of variation for total length was 9.20 per cent. This value rose so that by the end of the infection it was as high as 12.36 per cent. Here again we have an increase in the coefficient of variation which is undoubtedly significant from a statistical standpoint $[(12.36 \pm .59) - (9.20 \pm .44) = (3.16 \pm .74)]$,

TABLE 5.

Mean, standard deviation, and coefficient of variation for total length, and the number of trypanosomes per cmm. of blood at 4 intervals during the infection with T. rhodesiense in rat 703.

Each statistical constant is based on the measurement of 100 individuals, 400 trypanosomes having been measured during this infection. See Fig. 6 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	1 day 20 hrs.	2 days 20 hrs.	3 days 20 hrs.	5 days 20 hrs.
Mean length	25.733 ±.159	26.418	27.328	26.196
Standard deviation	2.368 ±.112	2.479	3.284	3.238
Coefficient of variation	9.20 ±.44	9.38	12.01	12.36
Number of trypanosomes per cmm. of blood	31,000	198,000	702,000	3,158,000

indicating that the parasites multiplied more rapidly at the end of the infection than at the beginning. We are, therefore, justified in concluding that not only does the host not build up any resistance which

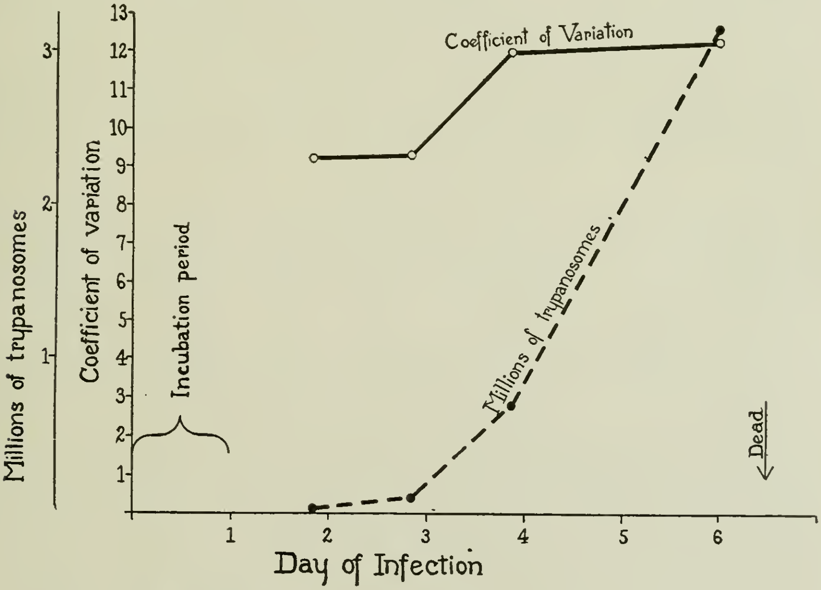

FIG. 6. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection with *T. rhodesiense* in rat 703.

retards the rate of reproduction, but there is some evidence that the parasites actually multiply faster as the infection progresses.

An examination of Table 5 and Fig. 6 also indicates that there is very little, if any, resistance which results in the destruction of the parasites after they are formed. These numbered 31,000 on the first day, and steadily increased so that they numbered 3,158,000 a few hours before the death of the host.

Rat 705.

Protocol 705. Culture rat infected with *T. rhodesiense.* Injected intraperitoneally with 0.5 c.c. of a 1–200 dilution of blood from a very heavily infected rat, on May 3, 1921 at 2:00 P.M. Organisms first demonstrated in the blood on May 5. Rat was dead by May 12. Calculations of the coefficient of variation for total length, based on the measurement of 100 individuals, and estimations of the number of organisms per cmm. of blood were made 5 times during the course of the infection on the dates shown in Fig. 7 and Table 6.

Incubation period was 1 day 18 hours.

Duration of the infection was approximately 7½ days.

TABLE 6.

Mean, standard deviation, and coefficient of variation for total length, and the number of trypanosomes per cmm. of blood on 5 days during the course of the infection with T. rhodesiense in rat 705.

Each statistical constant is based on the measurement of 100 individuals, 500 trypanosomes having been measured during this infection. See Fig. 7 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	2 days.	3 days.	5 days.	6 days.	7 days.
Mean length	25.709±.115	26.971	27.745	26.195	25.661
Standard deviation	2.304±.109	2.723	3.244	2.641	2.451
Coefficient of variation	**8.96 ±.43**	**10.09**	**11.69**	**10.08**	**9.55**
Number of trypanosomes per cmm. of blood	**31,000**	**506,000**	**1,225,000**	**1,303,000**	**3,308,000**

This infection was also of the acute type and was so similar to the two preceding infections that we will simply call attention to the results given in Table 6 and Fig. 7, and will not discuss them in detail. There is one point in regard to the coefficient of variation for total length, however, which should be pointed out. An examination of the data indicates that this probably remained constant throughout the course of the infection, and did not increase as was the case in rats 619 and 703. It is true that it was 8.96 per cent. on the second day and rose to the high value of 11.69 per cent. on the fifth day. We believe however, that these fluctuations, as great as they may at first seem, are merely due to experimental errors in technique, and that the true value of the coefficient of variation was approximately 10 per cent. throughout the course of the infection. This contention is supported by the fact that the high value reached on the fifth day (11.69 ± .56 per cent.) does not show a significant increase from the statistical standpoint over the preceding (10.09 ± .48 per cent.) or succeeding (10.08 ± .48 per cent.) determination. Its difference from the preceding measurement is 1.60 ± .74 which is only two times its probable error, and its difference from the succeeding measurement is 1.61 ± .74 which is also only two times its probable error. In any case, there is no evidence that the host has built up a resistance of either type to this trypanosome infection.

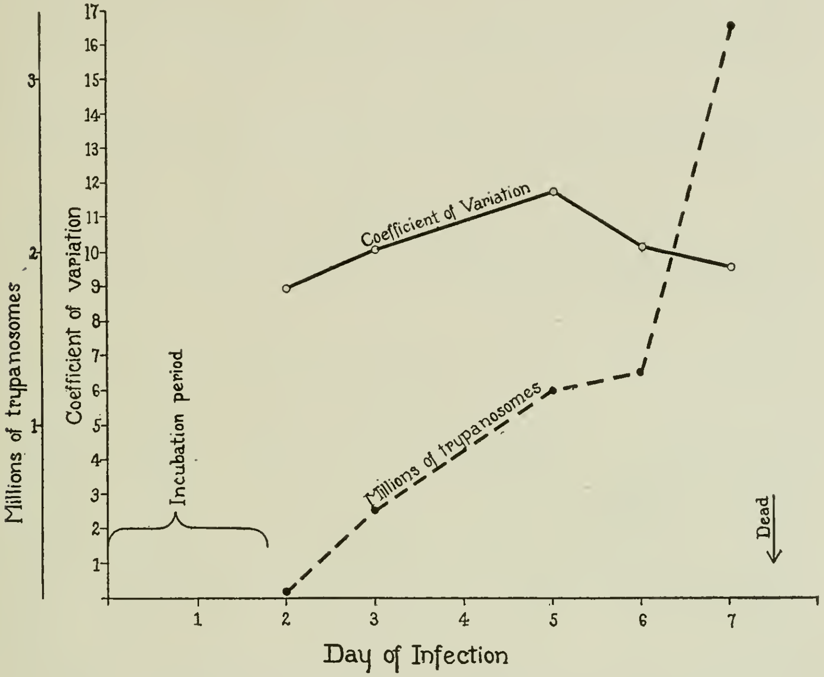

FIG. 7. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection with *T. rhodesiense* in rat 705.

Rat 709.

Protocol 709. Culture rat, infected with *T. rhodesiense.* Injected intraperitoneally with 0.5 c.c. of a 1–200 dilution of blood from a very heavily infected rat, on May 11, 1921, at 2:00 P.M. Organisms first demonstrated in the blood on May 13, at 2:00 P.M. Rat was dead by May 25. Calculations of the coefficient of variation for total length, based on the measurement of 100 individuals, and estimations of the number of organisms per cmm. of blood were made 9 times during the course of the infection, on the dates shown in Fig. 8 and Table 7.

Incubation period was 2 days.

Duration of the infection was approximately 13½ days.

TABLE 7.

Mean, standard deviation, and coefficient of variation for total length, and the number of trypanosomes per cmm. of blood at 9 intervals during the infection with T. rhodesiense in rat 709.

Each statistical constant is based on the measurement of 100 individuals, 900 trypanosomes having been measured during this infection. See Fig. 8 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	2 days 22 hrs.	4 days 20 hrs.	5 days 20 hrs.	7 days 1 hr.	7 days 22 hrs.
Mean length	26.509±.163	26.956	25.485	24.489	24.744
Standard deviation	2.430±.115	3.301	2.934	2.456	2.569
Coefficient of variation	9.16 ±.44	12.24	11.51	10.02	10.38
Number of trypanosomes per cmm. of blood	88,000	750,000	698,000	850,000	631,000

Interval after injection.	8 days 21 hrs.	9 days 21 hrs.	11 days 20 hrs.	13 days 2 hrs.
Mean length	23.894	24.603	25.445	24.575
Standard deviation	2.259	2.432	2.846	2.665
Coefficient of variation	9.45	9.88	11.18	10.84
Number of trypanosomes per cmm. of blood	956,000	613,000	1,867,000	2,388,000

This infection is interesting because it lasted twice as long as the previous infections and may, therefore, give us some insight into why some rats live longer with an infection of these pathogenic trypanosomes than others. The coefficient of variation for total length was 9.16 per cent. on the first day that the parasites appeared in the blood, and it stayed somewhere in the neighborhood of 10 per cent. for the rest of the infection. We do find that the value on the first determination (9.16 ± .44) is significantly lower than that at the second determination (12.24 ± .59). This difference is 3.08 ± .73 and is about four times its probable error. This second determination is, however, the only one which differs significantly from the remaining constants obtained, and we seem justified, therefore, in concluding that the coefficient of variation for total length, and, in consequence, the rate of multiplication of the trypanosomes remains constant throughout the infection.

Turning now to a consideration of the changes in the numbers per cmm., we find a different picture than in the infections of shorter duration. During the first days of the infection the number of parasites rose rapidly until it reached 750,000 per cmm. by the fifth day. Instead of increasing continuously, as in the previous infection, they then remained practically constant in number until the tenth day, when there were 613,000 per cmm. On the tenth day they began to increase again and numbered 2,388,000 per cmm. shortly before the death of the host. We have, therefore, clear evidence of a resistance being developed between the fifth and tenth days of the infection, which was directed solely toward the destruction of the parasites, and was apparently without effect upon the rate of reproduction of

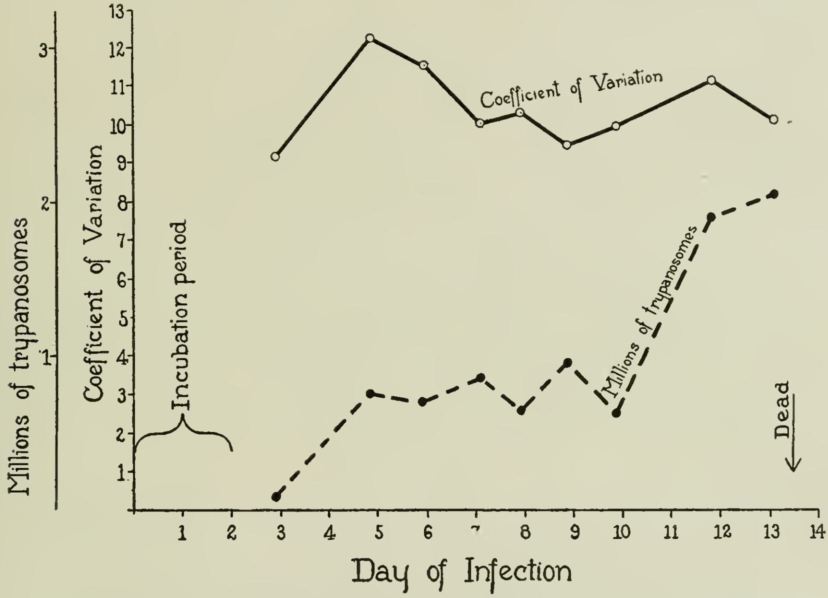

FIG. 8. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection with *T. rhodesiense* in rat 709.

the organisms which were not killed. It also seems quite probable that this five-day period of resistance was the underlying factor in the increased length of this infection. If this period be removed from our figure and table, our data present a picture almost exactly similar to the ones described for the shorter infections.

C. Pathogenic infection of the relapsing type. Of the trypanosomes which we have studied, only one, namely *T. equinum,* produces a true relapsing infection in the rat. Our general experience with this infection in the rat is closely similar to that of Pearce and Brown (1918). We have, however, worked up the data of only one infection of this type.

T. equinum: rat 729.

Protocol 729. Culture rat, infected with *T. equinum.* Injected intraperitoneally with 1 c.c. of a 1–200 dilution of blood from a lightly infected rat, on October 30, 1921. Organisms first demonstrated in the blood on November 2. Rat was dead by November 9, at 9:00 A.M. Calculations of the coefficient of variation for total length, based on the measurement of 100 individuals in each case, and estimations of the number of organisms per cmm. of blood, made 12 times during the course of the infection, on the dates shown in Fig. 9 and Table 8. After the trypanosomes first appeared in the blood they disappeared completely from the peripheral circulation on two occasions during the course of the infection, and could not be found on November 3 at 3:00 P.M., or on November 6, at 9:00 A.M. and 3:00 P.M. These two intervals are indicated in Fig. 9.

Incubation period was 3 days.

Duration of the infection was approximately 10½ days.

TABLE 8.

Mean, standard deviation, and coefficient of variation for total length at 12 intervals and the number of trypanosomes per cmm. of blood at 15 intervals during the course of the infection with T. equinum in rat 729.

Each statistical constant is based on the measurement of 100 individuals, 1,200 trypanosomes having been measured during this infection. See Fig. 9 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	3 days.	3 days 12 hrs.	4 days.	4 days 12 hrs.	5 days.	5 days 12 hrs.	6 days.	6 days 12 hrs.
Mean length....	21.719±.139	21.412	21.677	......	21.222	21.694	21.490	23.004
Standard deviation.........	2.073±.098	1.790	1.803	......	2.141	2.004	2.062	2.626
Coefficient of variation.....	9.54 ±.45	8.36	8.31	......	10.09	9.23	9.59	11.41
Number of trypanosomes per cmm. of blood.	4,000	48,000	73,000	000	800	3,000	8,000	9,000

Interval after injection.	7 days.	7 days 12 hrs.	8 days.	8 days 12 hrs.	9 days.	9 days 12 hrs.	10 days.
Mean length....	......	......	22.263	22.373	22.019	22.233	21.654
Standard deviation..........	......	......	2.083	2.220	2.078	2.315	2.346
Coefficient of variation.....	......	......	9.35	9.92	9.44	10.41	10.83
Number of trypanosomes per cmm. of blood.	000	000	483,000	1,171,000	3,436,000	4,869,000	9,707,000

An examination of the data in Fig. 9 and Table 8 indicates that there were no progressive changes in the rate of reproduction of the trypanosomes, as indicated by the coefficient of variation. This remained somewhere in the neighborhood of 10 per cent. throughout the entire infection. The number of the trypanosomes per cmm., however, showed some very marked fluctuations. They appeared in the blood three times during the course of the infection, and disappeared twice. During their first appearance they reached 73,000 per cmm. (the

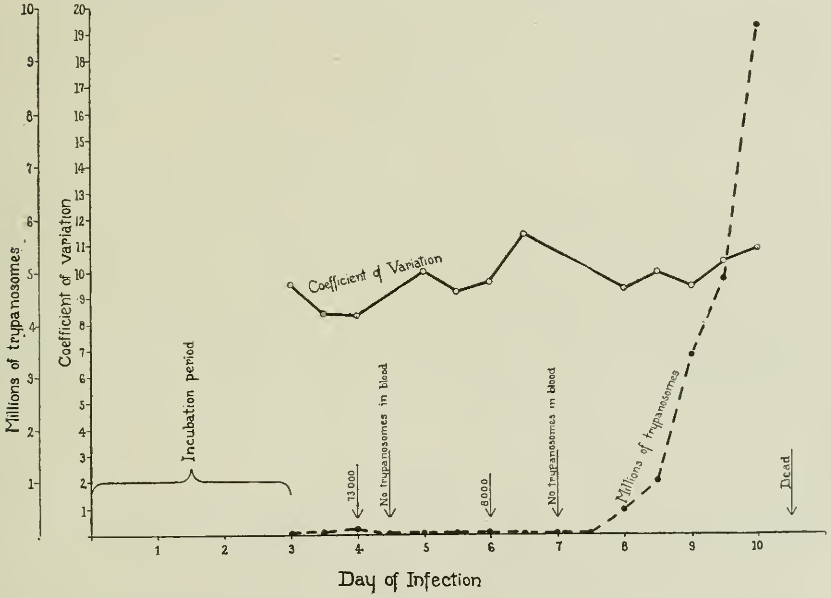

FIG. 9. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection with *T. equinum* in rat 729.

highest number), during their second 9,000 per cmm., and during their third 9,707,000 per cmm., culminating in death. These two complete disappearances of the organisms from the blood indicates the development of a resistance which destroys the organisms without any marked influence on their rate of multiplication.

The Mouse as a Host of Trypanosome Infection.

We have been unable to obtain *T. duttoni* which is a non-pathogenic trypanosome of mice, very similar to *T. lewisi* of the rat. Roudsky (1911), by a special treatment of *T. lewisi,* has been able to grow this organism in the mouse, in which case it develops into a lethal parasite. It would be very interesting to compare the condition of affairs in *T. lewisi,* when it is pathogenic and when it is non-pathogenic, but we have been unable to do this. The following two infections in the mouse are, perhaps, our best examples of infections in which the host builds up no resistance of either type.

A. Pathogenic infection of the continuous type.

T. brucei: mouse 728.

Protocol 728. Culture mouse, infected with *T. brucei.* Injected intraperitoneally with 0.75 c.c. of a 1–200 dilution of blood from a lightly infected guinea pig, on November 28, 1921, at 8:30 A.M. Organisms first demonstrated in the blood on November 29, at 9:00 A.M. Mouse was dead on December 2, at 9:00 A.M. Calculations of the coefficient of variation for total length, based on the measurement of 100 individuals in each case, and estimations of the number of organisms per cmm. of blood were made 6 times during the course of the infection, as is shown in Table 9 and Fig. 10.

Incubation period was 1 day.

Duration of the infection was approximately 3¾ days.

TABLE 9.

Mean, standard deviation, and coefficient of variation for total length, and the number of trypanosomes per cmm. of blood at 6 intervals during the infection with T. brucei in mouse 728.

Each statistical constant is based on the measurement of 100 individuals, 600 trypanosomes having been measured during this infection. See Fig. 10 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	1 day 2 hrs.	1 day 12 hrs.	2 days.	2 days 12 hrs.	3 days.	3 days 12 hrs.
Mean length	22.554±.125	22.336	22.793	22.213	23.005	22.480
Standard deviation	1.865±.088	2.224	2.261	2.247	2.101	2.267
Coefficient of variation	8.27 ±.39	10.05	9.92	10.11	9.13	10.08
Number of trypanosomes per cmm. of blood	900	4,000	10,000	93,000	409,000	4,103,000

An examination of Fig. 10 and Table 9 shows that, with the exception of the first day after the trypanosomes appeared in the blood, the coefficient of variation remained fairly constant at about 10 per cent. Even the difference between the low value of the first determination (8.27 ± .39) and the highest value obtained (10.11 ± .48) is only 1.84 ± .62, which is barely significant from a statistical standpoint. The number of trypanosomes per cmm. of blood was 900 at their first determination and rose to 4,103,000 before the death of the animal. An examination of the data shows, moreover, that this increase in numbers followed closely a curve of geometrical progression. This is the result one would expect if the rate of reproduction of the organisms remained constant, and if the host did not destroy many of them. In other words, there is no evidence of any resistance being built up either affecting the rate of reproduction or destroying the organisms.

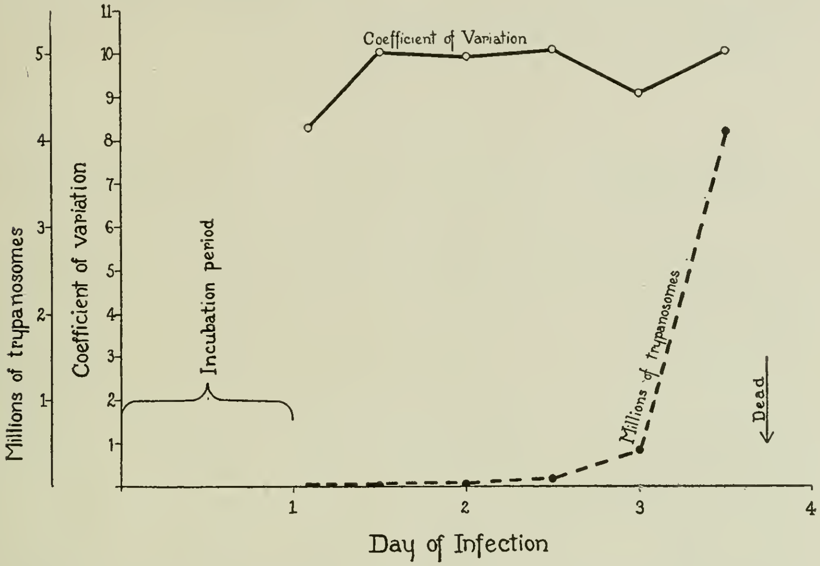

FIG. 10. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection with *T. brucei* in mouse 728.

T. rhodesiense: mouse 724.

Protocol 724. Culture mouse, infected with *T. rhodesiense.* Injected intraperitoneally with 1 c.c. of a 1–200 dilution of blood from a lightly infected rat, on

October 11, 1921, at 12 M. Organisms first demonstrated in the blood on October 15, at 9:00 A.M. Mouse was dead on October 19, at 9:00 A.M. Calculations of the coefficient of variation for total length, based on the measurement of 100 individuals in each case, and estimations of the number of trypanosomes per cmm. of blood were made 8 times during the course of the infection, as is shown in Table 10 and Fig. 11.

Incubation period was 3 days, 21 hours.

Duration of the infection was approximately 8 days.

TABLE 10.

Mean, standard deviation, and coefficient of variation for total length, and the number of trypanosomes per cmm. of blood at 8 intervals during the course of the infection with T. rhodesiense in mouse 724.

Each statistical constant is based on the measurement of 100 individuals, 800 trypanosomes having been measured during this infection. See Fig. 11 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	4 days 3 hrs.	4 days 10 hrs.	4 days 21 hrs.	5 days 9 hrs.
Mean length	26.679±.159	26.490	27.064	27.008
Standard deviation	2.367±.112	2.503	2.519	2.827
Coefficient of variation	8.87 ±.42	9.45	9.31	10.46
Number of trypanosomes per cmm. of blood	2,000	3,000	12,000	89,000

Interval after injection.	5 days 23 hrs.	6 days 9 hrs.	6 days 21 hrs.	7 days 9 hrs.
Mean length	25.848	26.337	26.978	26.432
Standard deviation	2.637	2.474	2.426	2.664
Coefficient of variation	10.20	9.39	8.99	10.07
Number of trypanosomes per cmm. of blood	243,000	305,000	1,344,000	6,526,000

The data for this mouse, which are contained in Fig. 11 and Table 10, lead to conclusions similar to those of the preceding infection. While the coefficient of variation showed more marked fluctuations, its true value probably lay between 9 and 10 per cent. throughout the course of the infection. That the coefficient of variation did remain constant is supported by the fact that the fluctuations from day to day are of no statistical significance. Take, for example, the large fluctuation between the constants obtained four days and twenty-one hours after the beginning of the infection (9.31 ± .44) and that obtained five days and nine hours afterwards (10.46 ± .50). The difference between these constants is 1.15 ± .67 which is less than twice the probable error, and cannot be considered of statistical importance. These considerations practically demonstrate that the rate of reproduction of the parasites remained constant throughout the infection.

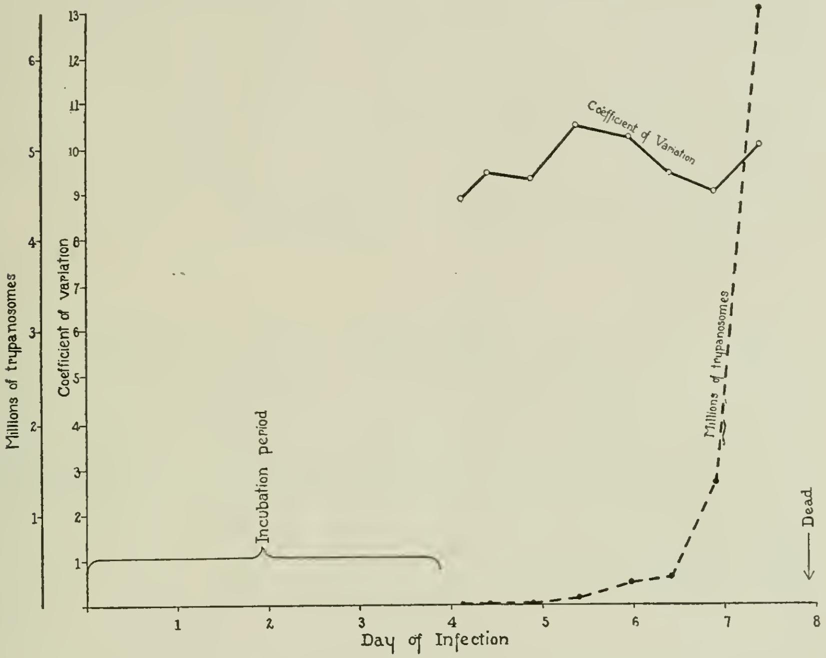

FIG. 11. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection with *T. rhodesiense* in mouse 724.

We also find that the number of parasites per cmm. of blood steadily increased from 2,000 per cmm. when the first determination was made, to 6,526,000 per cmm. just before death. Not only was this increase steady, but the type of increase was closely similar to a geometrical progression, and, therefore, indicates that the host destroyed very few, if any, of the parasites.

To sum up, then, the data for both mice indicate that no resistance is developed, either toward a retardation of the reproduction of the parasites or toward their destruction, after they are formed.

The Dog as a Host of Trypanosome Infection.

We know of no non-pathogenic trypanosome of the dog. The pathogenic African species in the dog present, in our experience, an entirely different type of infection than is observable in other laboratory animals. After the trypanosomes appear in the blood, they rapidly increase in numbers and then decrease, the host dying at the beginning of the second rise in numbers. While the decline in numbers can hardly be explained other than by their destruction by the host, the question still remains, does the host also produce an effect on the rate of reproduction of the parasites. An account of two cases of experimental trypanosomiasis in the dog follows.

A. Pathogenic infections of the relapsing type.

T. brucei: dog 713.

Protocol 713. Very young female puppy, weight 550 grams, infected with *T. brucei.* Injected intraperitoneally with 4 c.c. of a 1–50 dilution of blood from a lightly infected guinea pig, on July 9, 1921. Organisms first demonstrated in the blood on July 13. Dog was dead by July 21. Calculations of the coefficient of variation for total length, based on the measurement of 100 individuals in each case, and estimations of the number of organisms per cmm. of blood were made 8 times during the course of the infection, on the dates shown in Fig. 12 and Table 11.

Incubation period was 4 days.

Duration of the infection was approximately 11½ days.

TABLE 11.

Mean, standard deviation, and coefficient of variation for total length, and the number of trypanosomes per cmm. of blood on 8 days during the infection with T. brucei in dog 713.

Each statistical constant is based on the measurement of 100 individuals, 800 trypanosomes having been measured during this infection. See Fig. 12 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	4 days.	5 days.	6 days.	7 days.
Mean length	24.128±.165	24.434	24.750	26.069
Standard deviation	2.448±.116	2.496	2.438	2.544
Coefficient of variation	**10.14 ±.48**	**10.21**	**9.85**	**9.76**
Number of trypanosomes per cmm. of blood	**2,000**	**32,000**	**247,000**	**220,000**

Interval after injection.	9 days.	10 days.	10 days 6 hrs.	11 days.
Mean length	25.091	24.265	24.293	24.238
Standard deviation	2.435	3.315	2.665	2.208
Coefficient of variation	9.70	13.66	10.97	9.11
Number of trypanosomes per cmm. of blood	46,000	5,000	3,000	12,000

The coefficient of variation on the first day after the trypanosomes appeared in the blood (Fig. 12 and Table 11) was 10.14 per cent. It remained very uniform in value until the tenth day, when it rose to 13.66 per cent. The difference in these two constants, (13.66 ± .66) — (10.14 ± .48) = (3.52 ± .82), is approximately four times its probable error, and is, therefore, significant from a statistical standpoint. Such an increase in the coefficient of variation may be due to: first, an experimental error; second, an increase in the rate of reproduction of the parasites; or third, a possibility which is discussed on page 311, namely,

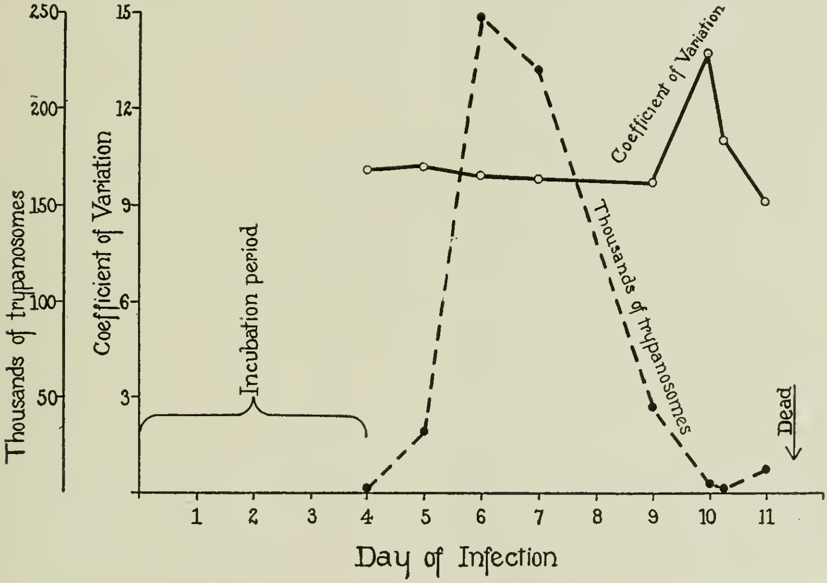

FIG. 12. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection with *T. brucei* in dog 713.

a production of abnormalities in the trypanosomes due to some adverse environmental factor, such as a trypanolytic antibody. Let us consider the last possibility first. There probably is a trypanolytic antibody formed during the infection, because this is the most plausible way to account for the partial disappearance of the trypanosomes from the blood. This will hardly account for this increase in variability, however, because it is not noticeable in any of the other infections in which a similar antibody is probably formed and, if it were true in this case, we would expect the increase in variability to begin when the organisms first begin to disappear from the blood, namely, on the sixth day. The increase, then, is due to either one of the first two possibilities. From our standpoint, however, this is of little importance, because the host certainly does not build up a resistance which *retards* the rate of reproduction, for the coefficient of variation shows no tendency to decrease in value.

While there is no evidence of a retardation in the reproduction of the parasites, there is evidence of their partial destruction. Between the fourth and sixth days their numbers mounted to 247,000 per cmm. They then rapidly disappeared from the blood, so that there were only 3,000 on the tenth day of the infection. During the next twenty-four hours, they again began to increase, and reached 12,000 shortly prior to the animal's death.

T. rhodesiense: dog 714.

Protocol 714. Very young female puppy, weight 400 grams, infected with *T. rhodesiense.* Injected intraperitoneally with 2 c.c. of a 1–50 dilution of blood from a heavily infected rat, on July 9, 1921. Organisms first demonstrated in the blood on July 11. Dog died on July 18, at 11:00 A.M., the last slides being made one hour before death. Calculations of the coefficient of variation for total length, based on the measurement of 100 individuals in each case, and estimations of the number of organisms per cmm. of blood were made 5 times during the course of the infection on the dates shown in Fig. 13 and Table 12.

Incubation period was 2 days.

Duration of the infection was 9 days.

The coefficient of variation for total length (Fig. 13 and Table 12) remained constantly around the value of 9 per cent. throughout the course of this infection. There were indeed no fluctuations which were of statistical significance. While it is unfortunate that no determination of the number of organisms per cmm. was made on the eighth day, it can be seen that the general nature of the changes in the number of parasites was similar to those found in dog 713. Between the fourth and sixth day of the infection, the number of trypanosomes rose until they were 94,000 per cmm. They then dropped to 51,000 on the seventh day and rose again to 61,000 on the ninth day, one hour prior to death.

TABLE 12.

Mean, standard deviation, and coefficient of variation for total length, and the number of trypanosomes per cmm. of blood on 5 days during the course of the infection with T. rhodesiense, in dog 714.

Each statistical constant is based on the measurement of 100 individuals, 500 trypanosomes having been measured during this infection. See Fig. 13 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	4 days.	5 days.	6 days.	7 days.	9 days.
Mean length	26.207±.154	26.492	25.983	24.899	23.612
Standard deviation	2.294±.109	2.388	2.525	2.270	2.227
Coefficient of variation	8.75 ±.42	9.01	9.75	9.11	9.59
Number of trypanosomes per cmm. of blood	7,000	41,000	94,000	51,000	61,000

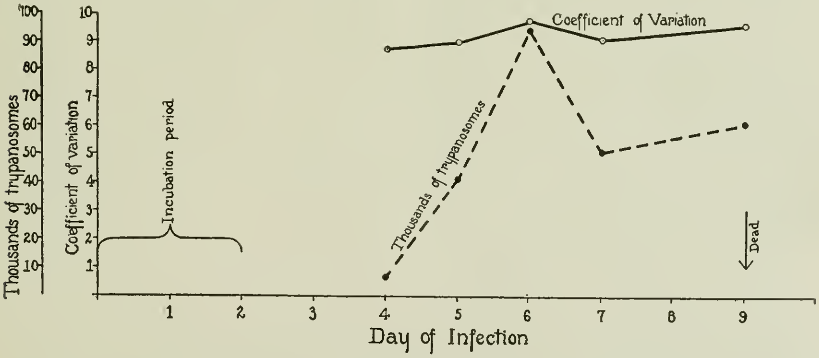

FIG. 13. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection with *T. rhodesiense* in dog 714.

To sum up, then, the results from these two dogs indicate that, while there is no resistance that affects the rate of reproduction, there is a marked resistance developed which is directed toward a destruction of the parasites after they are formed. It is significant to note that the development of this resistance apparently had little or no connection with the time of death. While the death of both animals took place when the trypanosomes were actually increasing in number, the number of parasites was low in comparison to the number present at the peak of the infection. This is in marked contrast to the condition of affairs in rats and mice (see especially the discussion under rat 709).

The Guinea Pig as a Host of Trypanosome Infection.

One of the best examples of relapsing types of infection is found when the pathogenic African trypanosomes are grown in the guinea pig. They are generally of a more chronic type than the infection in any of the previous hosts considered. A description of their general character can be found in Pearce and Brown (1918). The following experiments give an account of two of these infections.

A. Pathogenic infections of a relapsing type.

T. rhodesiense: guinea pig 719.

Protocol 719. Guinea pig, infected with *T. rhodesiense.* Injected intraperitoneally with 2 c.c. of a 1–200 dilution of blood from a heavily infected rat, on July 13, 1921. Organisms first demonstrated in the blood on July 18. Guinea pig was dead on August 25, at 9:00 A.M. Calculations of the coefficient of variation for total length, based on the measurement of 100 individuals in each case, and estimations of the number of organisms per cmm. of blood were made 19 times during the course of the infection, as is shown in Fig. 14 and Table 13. From the 5th to the 23d day of the infection only one or two trypanosomes could be found in fresh blood preparations. The infection during this period was so light that the organisms could be neither counted nor drawn.

Incubation period was 5 days.

Duration of the infection was approximately 42½ days.

This infection was a fairly long one, lasting for forty-three days (Fig. 14 and Table 13). After an incubation period of five days, a single trypanosome was demonstrated in the blood. From the fifth to the twenty-third day, one or two organisms could be found in fresh blood preparations, but they were never in great enough numbers, either to obtain a sample for the computation of the coefficient of variation, or to get an estimation of their numbers. This period of the infection undoubtedly represents a period of resistance by the host, but we have no way of telling whether the failure of the parasites to increase in number was due to a period of low reproductive activity or to the destruction of a large part of their numbers by the host.

TABLE 13.

Mean, standard deviation, and coefficient of variation for total length, and the number of trypanosomes per cmm. of blood on 19 intervals during the infection with T. rhodesiense in guinea pig 719.

For the first 17 days after the trypanosomes appeared in the blood, they were too few in number to count or measure. Each statistical constant is based on the measurement of 100 individuals, 1,900 trypanosomes having been measured during this infection. See Fig. 14 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	23 days.	24 days.	25 days.	26 days.
Mean length	26.132±.169	26.154	26.445	27.060
Standard deviation	2.517±.120	2.463	2.318	2.470
Coefficient of variation	9.63 ±.46	9.41	8.76	9.12
Number of trypanosomes per cmm. of blood	5,000	12,000	12,000	23,000

Interval after injection.	27 days.	28 days.	29 days.	30 days.	31 days.
Mean length	27.195	27.126	27.267	26.623	26.484
Standard deviation	2.771	2.689	3.057	2.511	2.523
Coefficient of variation	10.19	9.91	11.21	9.43	9.52
Number of trypanosomes per cmm. of blood	59,000	34,000	32,000	18,000	18,000

Interval after injection.	32 days.	34 days.	35 days.	36 days.	37 days.
Mean length	27.260	26.980	27.730	28.991	29.097
Standard deviation	3.060	2.719	3.019	2.996	3.158
Coefficient of variation	11.22	10.08	10.89	10.33	10.85
Number of trypanosomes per cmm. of blood	12,000	17,000	49,000	201,000	174,000

Interval after injection.	38 days.	39 days.	40 days.	41 days.	42 days.
Mean length	26.269	26.585	26.718	25.889	26.078
Standard deviation	3.234	3.086	3.217	2.972	2.646
Coefficient of variation	12.31	11.61	12.04	11.48	10.14
Number of trypanosomes per cmm. of blood	173,000	158,000	143,000	65,000	54,000

From the twenty-third day until the end of the infection, we were able to make daily determinations both of the coefficient of variation for total length and of the number of organisms per cmm. of blood. While there were many fluctuations in the former constant, a few of which are significant from a statistical standpoint, it remained fairly uniform around the value of 10 per cent. Barring the first chronic stage of the infection during which we have no data, we have no evidence that the host produces any marked effect on the rate of reproduction of the parasites.

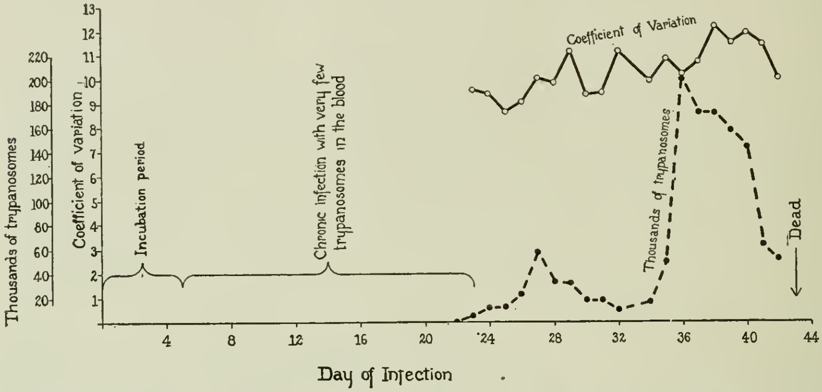

FIG. 14. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection of *T. rhodesiense* in guinea pig 719.

From the twenty-third to the twenty-seventh day, the parasites rapidly increased in number, so that there were 59,000 in the blood at the latter time. They then decreased until there were only 12,000 on the thirty-second day. A very much more rapid increase now set in, bringing the numbers up to 201,000 on the thirty-sixth day. They then decreased again so that there were only 54,000 on the forty-second day, the host dying while the parasites were on the decrease. We have, therefore, clear evidences that the host built up a resistance which caused two partial disappearances of the parasites from the blood, and which, in no way, was connected with the actual rate of reproduction of the parasites.

T. brucei: guinea pig 721.

Protocol 721. Guinea pig, infected with *T. brucei.* Injected intraperitoneally with 1 c.c. of a 1–50 dilution of blood from a lightly infected guinea pig, on July 14, 1921. Guinea pig was dead on August 10, at 8:30 A.M. Organisms first demonstrated in the blood on July 19. Calculations of the coefficient of variation for total length, based on the measurement of 100 individuals in each case, and estimations of the number of organisms per cmm. of blood were made 12 times during the course of the infection, as is shown in Table 14 and Fig. 15. After the trypanosomes first appeared in the blood they disappeared completely from the peripheral circulation between the 7th and 12th days and between the 18th and 20th days of the infection, as is indicated in Fig. 15.

Incubation period was 5 days.

Duration of the infection was approximately 26½ days.

TABLE 14.

Mean, standard deviation and coefficient of variation for total length on 12 days and the number of trypanosomes per cmm. of blood on 20 days during the course of the infection with T. brucei in guinea pig 721.

Each statistical constant is based on the measurement of 100 individuals, 1,200 trypanosomes having been measured during this infection. See Fig. 15 for graph of the coefficient of variation and the number of trypanosomes per cmm. of blood.

Interval after injection.	6 days.	7 days.	8 days.	9 days.	10 days.	11 days.	12 days.
Mean length	23.874±.194	......	......	......	......	......	......
Standard deviation	2.888±.137	......	......	......	......	......	......
Coefficient of variation	12.09 ±.58	......	......	......	......	......	......
Number of trypanosomes per cmm. of blood	2,000	000	000	000	000	000	000

Interval after injection.	13 days.	14 days.	15 days.	16 days.	17 days.	18 days.	19 days.
Mean length	25.528	23.704	24.509	26.495	25.694	......	......
Standard deviation	2.490	2.761	2.501	2.673	2.475	......	......
Coefficient of variation	9.75	11.65	10.20	10.08	9.63	......	......
Number of trypanosomes per cmm. of blood	17,000	2,000	800	5,000	2,000	000	000

Interval after injection.	20 days.	21 days.	22 days.	23 days.	24 days.	25 days.	26 days.
Mean length	......	23.209	24.917	24.704	25.094	25.180	23.678
Standard deviation	......	2.133	2.631	2.287	2.383	2.360	2.471
Coefficient of variation	......	9.19	10.56	9.25	9.49	9.37	10.43
Number of trypanosomes per cmm. of blood	000	9,000	14,000	90,000	148,000	227,000	252,000

This infection with *T. brucei* in the guinea pig was closely similar to the one of *T. equinum* in the rat; the chief difference being in the length of the infection. Here again there is little evidence that there was any marked progressive change in the coefficient of variation for total length. The true value of this constant was about 10 per cent. throughout the course of the infection (Fig. 15 and Table 14). Between the fifth and sixth days of the infection the parasites increased, until they numbered 2,000 per cmm. of blood. They then disappeared from the peripheral circulation and were not found between the seventh and twelfth days. They reappeared on the thirteenth day and increased until their number was 17,000 per cmm. Here again, they diminished to 800 on the fifteenth day and rose to 5,000 on the sixteenth day. This was followed by a complete disappearance from the blood between the eighteenth and twentieth days. Their next appearance in the blood, however, was their last, and their numbers rapidly rose to 252,000 on the twenty-sixth day, just before the death of the guinea pig. In this infection, therefore, we have four periods of increase in the number of the parasites and three periods of decrease, two of which resulted in their complete disappearance from the peripheral circulation.

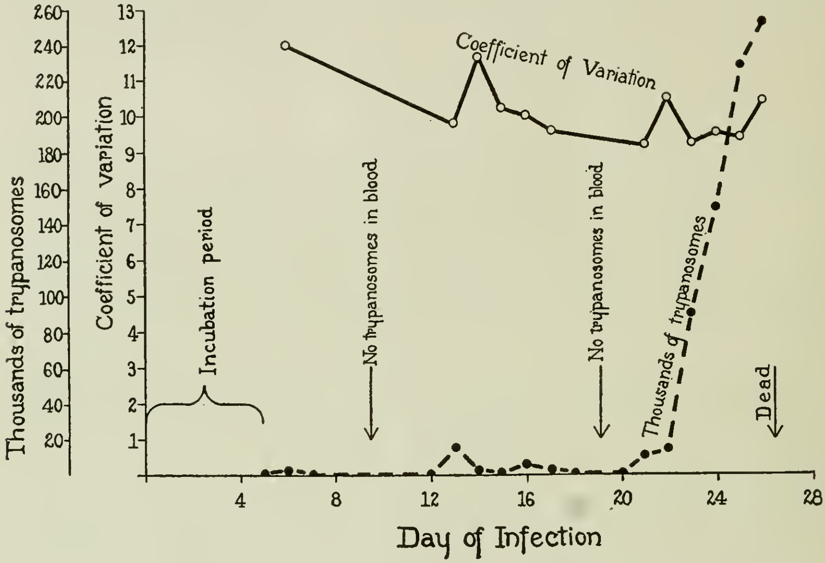

FIG. 15. Graph showing the coefficient of variation for total length (comparative measure of the rate of reproduction) and the number of trypanosomes per cmm. of blood, throughout the course of the infection of *T. brucei* in guinea pig 721.

To sum up, then, the results from both guinea pigs indicate that the partial or complete disappearance of the trypanosomes from the blood is associated with their actual destruction by the host, rather than with any retardation or inhibition of their reproductive rate.

These two guinea pig infections bring out a point which is often observed in the laboratory, namely, that the death of the guinea pigs is apparently not dependent upon the actual number of organisms per cmm. of blood. The death of the guinea pig may occur when the number of the organisms is at the highest point (pig 721) or it may occur when the organisms are comparatively very few in number and are actually disappearing from the blood (pig 719). The same condition of affairs, as we have already pointed out, holds for the dog, but it is not true for the rat or mouse. In the last two animals, death always occurs when the trypanosomes are most plentiful in the blood, and apparently never takes place until the host is suffering from an exceedingly high grade of blood infection.

THE COEFFICIENT OF VARIATION FOR SIZE AS A COMPARATIVE MEASURE OF THE RATE OF REPRODUCTION.

In the *Introduction* we gave a short account of the fundamental basis for using the coefficient of variation for some factor involving size as a comparative measure of the rate of reproduction or growth. We also pointed out its obvious advantage, viz., that it enabled us to estimate the rate of reproduction, even though most of the individuals resulting from this reproduction were killed. In other words, it is independent of numbers as long as we can measure enough individuals to form a sample of suitable size for statistical analysis. In presenting the preceding data we have assumed that it was a valid method for the estimation of the rate of reproduction of the trypanosomes. The establishment of its validity, however, is so fundamental from the standpoint of the present work that it seems advisable to discuss it again in this place. Such a discussion can be taken up under several heads.

A. *Rationale of its use.* As has already been pointed out, it is perfectly clear from an *a priori* standpoint that reproduction with its production of young forms and consequent series of growth stages must necessarily increase the coefficient of variation for size. Furthermore, it is also evident that this increase in variability is more or less proportional to the rate of the production of young forms, or, in other words, to the rate of reproduction. This reasoning should be valid for almost any animal. Take, for example, man. If we make a comparison of the variability of some measure of size, such as height, between a population in which no reproduction is taking place and one in which rapid reproduction is occurring, the former will consist largely of adults who will be more or less uniform in height, while the latter will consist of adults and children in all stages of growth and will exhibit a very much greater variability in height. It is also apparent that the greater the number of children (the higher the rate of reproduction) the greater will be the variability. In trypanosomes where each division results in the formation of one long and one short individual, the same reasoning will hold in regard to the variability in total length.

B. Observational evidence for trypanosomes. It must not be supposed, however, that our results are based entirely on this *a priori* reasoning, sound as it may seem. In the experiments on *T. lewisi* in the rat one can satisfy oneself of the general validity of the conclusions by an examination of the slides. Take, for example, rat 105. The slides on the fourth day of the infection contain many organisms in the process of division. These evidences of division decreased until one could never find any but typical " adult " trypanosomes, after the twenty-eighth day. In fact, our general results in regard to the rate of reproduction in *T. lewisi* have been known for some time, as is shown by the work of Minchin (1912) and Minchin and Thomson (1915). Previous investigators determined the length of the multiplicative stage of the infection by just such an examination of the slides as we have outlined above. In this connection Minchin and Thomson (1915) say: " So long as the trypanosomes are multiplying in the rat's blood, they are of various sizes, some of the ordinary, normal size, others very small, and others again much above the normal size. Marked variation in the size of the trypanosomes is a sure sign that multiplication is proceeding, even when actual division-stages are so scarce in the preparation that prolonged search is necessary in order to find them. As soon as the multiplication is ended the trypanosomes are all of one type and size, allowing for slight individual variations that are not perceptible without careful measurement; to such trypanosomes, the normal form of *T. lewisi* and the sole form occurring in the blood when once the multiplication is at end, we shall refer always as ' ordinary.' " We give this quotation from Minchin and Thomson not only to show that they knew the general facts which we have outlined in regard to the changes in the rate of reproduction throughout the course of infections of *T. lewisi* in the rat, but that they arrived at these conclusions by approximately the same method that we have used, namely, a comparison of the relative variability in size in the trypanosomes. Their method, however, was based purely on a personal estimation of the variability in size, whereas ours is based on the far more exact method of determining the coefficient of variation for total length.

While previous investigators have used approximately the same method as ours (although less exact) for estimating the changes of the rate of reproduction of trypanosomes throughout the course of an infection of *T. lewisi* in the rat, we know of no case where this has been used for the pathogenic trypanosomes. One might say that the method is valid for *T. lewisi*, because in these infections a comparison can be made between the trypanosomes when they are known to be reproducing rapidly and when all reproduction is known to have ceased, whereas in the pathogenic forms no such opportunity is offered, because the organisms reproduce continuously throughout the infection. In other words, we can prove that the method works for *T. lewisi*, while this is not so easy for pathogenic forms. For this reason we have undertaken to show that reproduction in the pathogenic forms, as well as in *T. lewisi*, must raise the coefficient of variation for total length. Our method for demonstrating this is based on the following facts: first, we know the general sequence of changes which take place when a trypanosome divides; second, if we measure a large number of trypanosomes and group them according to these various stages, we can show the alterations in size which result from division and its concomitant growth; third, knowing these facts, we should be able to ascertain whether or not reproduction in the pathogenic forms increases the coefficient of variation for total length, as it does in *T. lewisi*.

Let us first outline the sequence of changes during the process of division in a trypanosome. First, the blepharoplast divides (see Fig. 2), and this is followed shortly by the division of the parabasal body. After two parabasal bodies are formed, the nucleus divides, and then division of the body proceeds from the anterior to the posterior end. As we can not differentiate the blepharoplast from the parabasal body in ordinary work, we have to begin with the division of the latter. We have measured 260 trypanosomes from a slide made on the last day of the infection of *T. equiperdum*, in rat 619, and have separated these measurements into five groups which correspond to the stages in, what we might call, the life-history of the individual trypanosome. The individuals are placed in the separate groups, according to their stage in division as follows:* (1) all forms possessing a single parabasal body and nucleus, (2) forms possessing two parabasal bodies, but a single nucleus, (3) forms having two parabasal bodies and the nucleus in the process of division, (4) forms possessing two parabasal bodies and two nuclei, but showing no evidence of division of the body, and (5) forms with the body in actual process of division. It is to be emphasized that the first class, although it contains forms which are not dividing, is a very much more heterogeneous class than the others, and should show a greater range of variation. This follows from the fact that it contains not only "adult" forms, but the

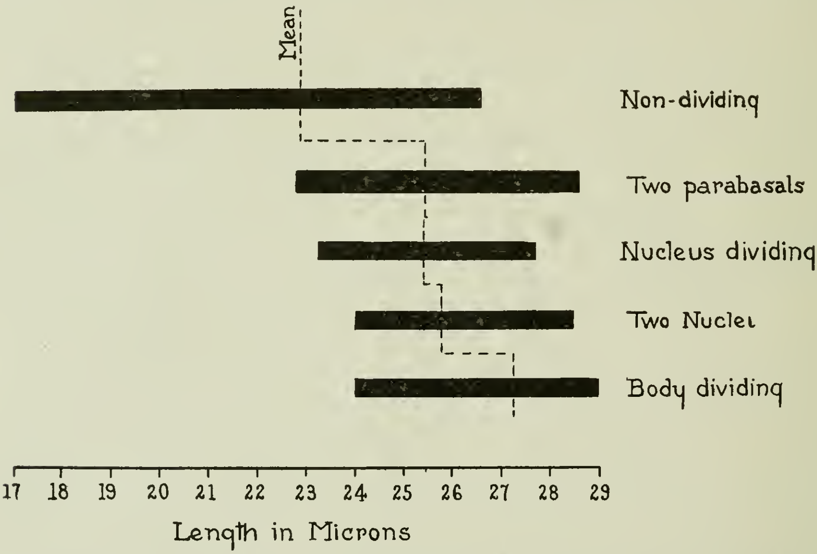

FIG. 16. Bar diagram showing the range of variation in total length between sets of *T. equiperdum* which (1) show no signs of division, (2) possess two parabasal bodies and one undivided nucleus, (3) possess two parabasal bodies and one nucleus in the process of division, (4) possess two parabasal bodies and two nuclei, and (5) show the body in actual process of division. The broken line indicates the mean of each group. For full explanation see text.

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* The 260 individuals do not represent a random sample of the general population, but each class is itself a random sample of the trypanosomes in that stage of division. In other words, the number of individuals in each class does not represent the true proportion of the individuals in that stage of division, as found in a random sample of the general population.

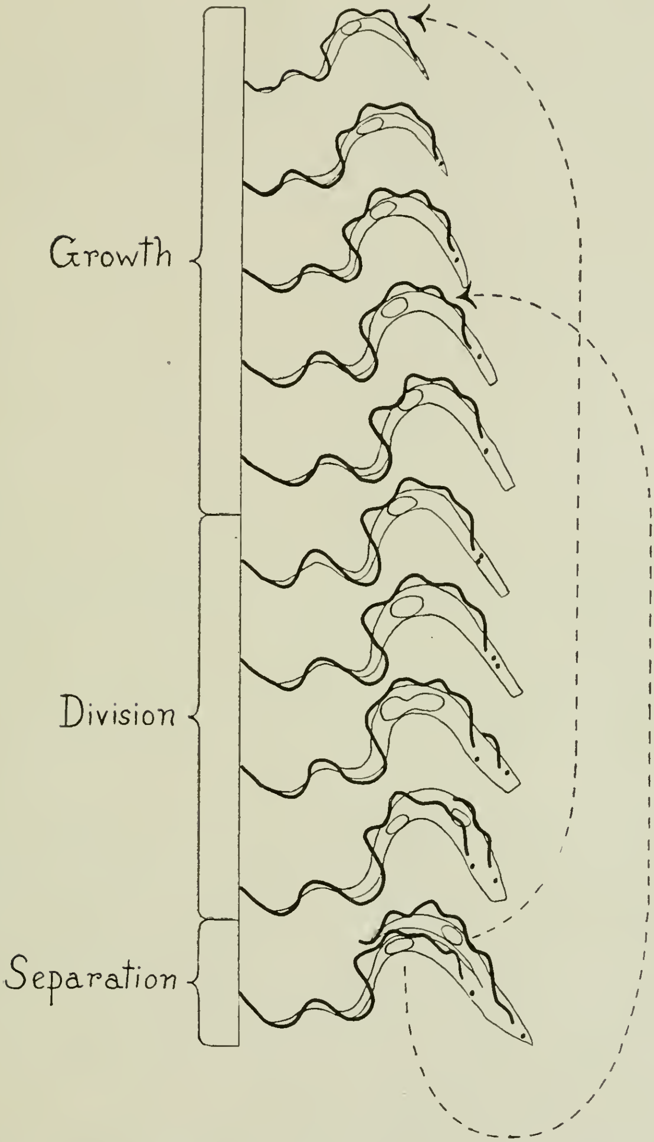

FIG. 17. Diagram representing the probable course of events in asexual reproduction in *T. equiperdum*. This diagram is based on the results given in the text and in Fig. 16.

young forms which result from the division of the fifth class, and all intermediate stages of growth.

The results of these measurements are shown graphically in Fig. 16. 125 individuals in class 1, containing both adults and young forms, ranged in size from 17.1 to 26.6 microns, with a mean of 22.9. In class 2, which contained 60 individuals, the range in total length was from 22.8 to 28.6 microns, with a mean of 25.5. Class 3, of 25 individuals, ranged from 23.2 to 27.7 microns, with a mean of 25.4. The range of size of classes 4 and 5 was closely similar to the two preceding classes, class 4 ranging from 24.0 to 28.5 microns, with a mean of 25.7, and class 5 ranging from 24.0 to 29.0 microns, with a mean of 27.3. It is very important to note that an examination of the individuals in class 5, which consisted of forms with their bodies actually dividing, shows that the division always produced one very short and one very long form. These data bring out the following facts: The mean length of class 1 is low, due to the fact that it contains so many young forms. Class 2, which contains those forms beginning to divide, shows a very much higher mean and a very much shorter range of variation than class 1. This demonstrates that all of the short forms produced by division grow to the "adult" size before they proceed to divide again. If this were not true, the range of variation in class 2 would more nearly approach that of class 1. A comparison of the mean length of classes 2 to 4 inclusive shows that there is no appreciable increase in the mean during the actual process of division. The large increase in mean in class 5 is to be explained by a shifting in the position of the two component forms, just prior to separation, rather than any actual growth. We have tried to show these facts in Fig. 17. Figure 18 shows the frequency distribution of class 1, containing a mixture of "adult" forms and young forms in all stages of growth as compared with the frequency distribution of classes 2 to 4 inclusive, containing "adults" only. As we would expect, the former shows a much higher degree of variation in total length. Class 5 was not included in this distribution because, as we have pointed out above, its difference in mean length (from classes 2 to 4) is not due to growth.

The demonstration that a pathogenic trypanosome in reproducing always produces one long and one short individual, and that the resultants of division always grow to the "adult" size before dividing again clearly indicates that reproduction in these forms, as well as in *T. lewisi,* increases the coefficient of variation for total length.

While the coefficient of variation for total length is as valuable an indicator of the rate of reproduction in the pathogenic forms as in *T. lewisi,* it is interesting to note that the actual changes in total length resulting from division are somewhat different in the two cases. The foregoing evidence indicates that in the pathogenic trypanosome, *T. equiperdum,* once an organism has grown to its "adult" size, it does not increase appreciably in length during the actual process of division. The higher variability resulting from division is due chiefly to growth of young forms. In *T. lewisi* we not only have this factor, but the "adult" trypanosomes apparently increase in length just prior to division. That this is true is indicated by a comparison of samples *A* and *B* in Fig. 1. Here it can be seen that one of the individuals in the rapidly reproducing population is considerably longer than any of the "adult" forms from the non-reproducing population.

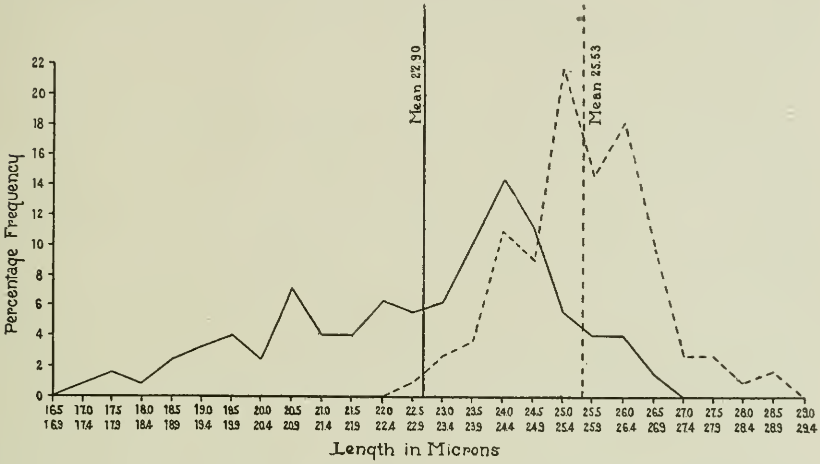

FIG. 18. Frequency polygon showing the variation of those forms which show no signs of division, and which include forms of all ages (solid line), as compared with those actually in the process of division which are "adults" (broken line).

*C. Other factors besides growth and reproduction which might affect the variability in size.** The question might be raised whether other factors beside growth and reproduction might not influence the coefficient of variation for total length. Investigators working with free-living species of protozoa have noted that such things as temperature, food supply, the addition of chemicals to the culture medium, etc. (see especially Erdmann, 1920) increase the variability in size of populations. Most of these environmental factors are known to affect the rate of reproduction, and it seems very probable that their effect on the variability in size may be due to their effect on the rate of reproduction. In any event most of these factors are usually constant from day to day for a parasite living in mammalian blood.

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* We have shown elsewhere (Taliaferro, 1921 *b*) that passage of *T. lewisi* through the invertebrate host (in contradistinction to direct passage through the vertebrate host) increases variability, and that this increase in variability is not due to any phenomenon of growth. This, however, has no bearing on the present paper where all work was done with trypanosomes in the vertebrate host.

There are, however, other environmental factors which might increase variability and size without increasing the rate of reproduction. Suppose, for example, that a trypanolytic antibody is formed within the host. In killing the organisms this lysin might produce many distortions in shape and size entirely independent of growth. In the numerous cases presented in the preceding experiments where the trypanosomes suddenly decreased in number or disappeared from the blood, such a trypanolytic antibody is probably formed and yet in none of these cases is there any evidence of an increase in the coefficient of variation for total length. The sudden rise of the coefficient of variation on the tenth day in dog 713 might be considered an exception to this statement. We do not think, however, that this increase in variability is due to the formation of a trypanolytic antibody because: first, it is the only case of such an increase among six experiments in which there is an effect on the number of trypanosomes and in which, presumably, a trypanolytic antibody is formed; and second, if this increase had been due to a trypanolytic antibody, it would have begun when the effect of the lytic agent first became apparent, i.e., on the seventh day.

The production of a trypanolytic antibody might conceivably affect the variability in size in just the opposite direction, in that it might lower the coefficient of variation due to the selective killing of trypanosomes of a specific size. It might, for example, kill only the young forms and leave the ''adults.'' If such selective killing occurred, we would not only expect a decrease in the coefficient of variation, but also a marked change in the mean size. It is evident that the latter is true because the mean would be lowered or raised according to whether the trypanolytic antibody selected the large ''adult'' forms or the small young forms. An examination of the data for the pathogenic forms reveals the fact that not only is the coefficient of variation for total length constant, but the mean length shows very few changes throughout the course of each infection.

While there is no evidence of any selective action on the part of the destructive agent for the pathogenic trypanosomes, this is not as apparent in the two infections with *T. lewisi.* In these infections we not only find changes in the coefficient of variation throughout the infection, but we also find changes in the mean length. Let us first consider the coefficient of variation. During the first part of the infection, this rapidly decreases in value. This decrease, however, cannot be due to the selective killing of the trypanosomes, because by far the greater part of the decrease takes place *before* the trypanosomes begin to disappear from the blood. The mean length (see especially rat 105) increases during the first part of the infection, but in this case the entire increase takes place before the trypanosomes begin to disappear. In both cases, therefore, the changes seem to be independent of the disappearance of the trypanosomes from the blood, and, therefore, independent of the action of the trypanolytic antibody. Finally, in this connection, it is well to repeat that our conclusions in regard to the rate of reproduction of *T. lewisi* in the rat, which were drawn from a study of the changes in the coefficient of variation for total length, have been checked and verified by numerous direct observations.

Discussion of Results.

A brief discussion of some of the fundamental factors involved in the resistance of a host to trypanosome infections, as well as the results obtained in our work, will enable us to understand more clearly the conclusions drawn in this paper. We have used the term resistance to denote collectively those factors, either active or passive, which operate adversely against the invading organism. Our particular interest in the resistance of the host to trypanosomes is in that resistance which develops after the organisms are established in the host. We have not taken into consideration any of the great questions of the resistance of various hosts to invasion. Considering the former type of resistance from the logical standpoint, it seems to us evident that this may be divided into those factors which tend to retard the rate of reproduction of the parasite and those which tend to actually destroy them after they are formed. Both of these factors will, of course, affect the number of organisms present in the blood at any given time. Taking these facts into consideration we have been able to differentiate between them by making frequent determinations of the number of organisms per cmm. of blood and of the coefficient of variation for total length, which gives us a comparative measure of the rate of reproduction of the trypanosomes, regardless of the number destroyed in consequence of resistance. The rationale of this general procedure is given on page 267 and a discussion of the validity of the coefficient of variation for total length as a comparative measure of the rate of reproduction is given on page 305.

Before leaving the question of the coefficient of variation for total length, it is well to call attention to the fact that in our tables we have given not only the actual coefficient, but the mean length and standard deviation from which the coefficient of variation was calculated. Incidentally, these tables contain some very valuable data in regard to the effect on total length, of growing the same strain of trypanosomes in different hosts. We will not consider this question in the present study, but will reserve it for a later paper.

Turning now to a consideration of the resistance which a host offers toward a trypanosome infection after the parasites have established themselves, we find that three different conditions have been brought out by the preceding experiments. In the first place, the highest grade of resistance was found in the non-pathogenic *T. lewisi* in the rat. Here the host builds up an ever increasing resistance which retards the rate of reproduction of the trypanosomes. This resistance is so pronounced that, during the last (or "adult") stage of the infection, the parasites exist in the blood in the so-called "adult" condition, without exhibiting any signs of reproduction or growth. We also find in this case that just as the resistance against the rate of reproduction is reaching its climax (viz., 10th day in rat 105), the host suddenly develops a resistance which destroys a majority of the organisms in the blood. The remaining parasites live in the blood for varying lengths of time (sometimes several months) until they are suddenly destroyed as the result of the final resistance which the host builds up against them. After their disappearance from the blood, the host is immune to another infection of *T. lewisi* (see Laveran and Mesnil, 1912). In the second place, we find just the opposite condition of affairs in most infections of rats and mice due to pathogenic trypanosomes. Here (with the exception of long infections, such as rat 709 and rat 729) we can find no evidence that any resistance is built up either toward the rate of reproduction or toward the destruction of the parasites, after they are formed. Once the trypanosomes establish themselves in the host, they reproduce and live in the blood unmolested. In some cases, indeed, there is evidence that they reproduce more rapidly as the infection progresses (rats 619 and 703). In the third place, we find in the relapsing type of infection (pathogenic trypanosomes in the dog and guinea pig and *T. equinum* in the rat) that no resistance is built up which retards the rate of reproduction of the trypanosomes, but a resistance does arise which destroys a large number of the parasites after they are formed.

The fact that resistance in the non-pathogenic *T. lewisi* produces two different effects whereas resistance in pathogenic species (if present at all) produces only one of these effects indicates that in the non-pathogenic forms there is either an entirely different type of mechanism of resistance, or that there is a different type of mechanism superimposed on the mechanism found in the pathogenic forms. It is interesting to note that Miss Robertson (1912) has brought forward considerable evidence to show that in *T. gambiense* in the monkey there is an endogenous cycle, in which phases of active reproduction alternate with phases of little or no reproduction. If this be true, it would indicate that this form in the monkey encounters much the same type of resistance as *T. lewisi* in the rat, except that in the latter case, when reproduction once ceases it never starts again. Miss Robertson's results indicate that *T. gambiense* in the monkey encounters a resistance midway between the type we have described in *T. lewisi* in the rat and the pathogenic species in the guinea pig. Such a result would not be at all surprising, and with the growing evidence that *T. gambiense* can exist in certain mammals as a non-pathogenic parasite it may be possible to find a host in which this trypanosome encounters the same type of resistance as *T. lewisi* in the rat.

In considering the resistance encountered by the pathogenic trypanosomes, the question immediately arises what effect the development of a resistance has on the time of death of the host. In the case of these trypanosomes in the rat we found one case (*T. rhodesiense* in rat 709) which strongly suggested that the development of a resistance tending to destroy the parasites actually prolonged the life of the host. In other words, there seems to be a definite connection in rats, and probably mice, between the number of organisms in the blood and the time of death of the host. This correlation does not, however, seem to hold good when the same trypanosomes are grown in the dog and guinea pig. In our two experiments on dogs, both animals died when the number of trypanosomes in the blood was very much less than it was at the peak of the infection. Of the two guinea pigs one died at a time when the number of trypanosomes in the blood was at its maximum height, whereas the other died when they were comparatively few in number. Of course, we can not say that the development of a resistance in the latter hosts did not lengthen the life of the animal. The results themselves, however, do not reveal any obvious connection between the two factors as was suggested in the case of *T. rhodesiense* in rat 709.

In our preceding discussion we have assumed that a disappearance of the trypanosomes from the peripheral blood indicates that they have been actually destroyed by the host and not that they have migrated into the tissues. As has already been pointed out, this seems to be a safe assumption to make, because Levaditi and McIntosh (1910) have demonstrated the presence of a trypanolytic antibody after the crises in such infections. Then, too, if the disappearance from the blood were to be explained by a migration into the tissues, there should be a great many more parasites in the tissues during the period of low blood infection. At the present time, there is not the slightest evidence that this is true. The same conclusion was reached by Miss Robertson (1912), although on slightly different premises. Of course, had we worked with *T. cruzi*, the condition of affairs would have been different, because this trypanosome shows an entirely different behavior and method of reproduction than do any of the other trypanosomes. In fact, the most typical form of this parasite, in the vertebrate host, is an almost true tissue-inhabiting Leishmania.

We feel that, in the present work, we have been able to introduce a new method of attack on some of the problems of resistance. This method, however, deals with the *effect* of the resistance, and not with its *mechanism*. What might constitute the most probable explanation of the effects which we have observed would necessitate a lengthy discussion. This, however, had best wait until we can do some actual experimental work on the question. We will point out, however, that the two different types of resistance in *T. lewisi* may be connected with the same fundamental mechanism.

In the future, we hope to continue the study of resistance and extend our method, if possible, to other infections. It is obvious that it can not be used unless accurate measurements of the size of the parasites can be made. This can be done for a number of protozoa, but it is impracticable for most of the bacteria. It is possible, however, that the spirochetes will lend themselves to this method of attack.

General Summary.

1. The conclusions reached in this paper are based on the study of 13 experimental infections with trypanosomes, divided as follows: *T. lewisi* in the rat (2), *T. equiperdum* in the rat (1), *T. equinum* in the rat (1), *T. brucei* in the dog (1), mouse (1), and guinea pig (1), and *T. rhodesiense* in the rat (3), mouse (1), dog (1), and guinea pig (1). In the study of these infections determinations of the number of trypanosomes per cmm. of blood were made on 125 days and drawings, measurements, and computations of statistical constants were made for 114 sets of 100 trypanosomes each.

2. The resistance which a host builds up toward a trypanosome infection may do one or both of two things: (*a*) it may retard the rate of reproduction of the parasites, or (*b*) it may destroy the parasites after they are formed.

3. These two conditions can be differentiated by ascertaining: (*a*) the changes in the number of parasites per cmm. of blood, supplemented by (*b*) the changes in the coefficient of variation for total length of the trypanosomes, which gives a comparative measure of their rate of reproduction, irrespective of the number destroyed in consequence of the resistance. These two determinations differentiate between the two types of resistance because:

The number of trypanosomes per cmm. of blood = the number produced by reproduction of the parasites — the number destroyed in consequence of the resistance.

4. The validity of the coefficient of variation for total length, as a measure of the rate of reproduction of trypanosomes, is discussed in detail on page 305. Its great value lies in the fact that it measures the rate of reproduction irrespective of the number destroyed by the resistance.

5. Three conditions have been found in regard to the resistance to trypanosome infections.

(*a*) In the non-pathogenic *T. lewisi* in the rat, there is a resistance built up which is directed both toward a retardation of the rate of reproduction of the parasites, and toward their actual destruction after they are formed (rats 105 and 116).

(*b*) In the continuous infections: *T. rhodesiense* (with the exception of the long infection in rat 709), *T. brucei, T. equiperdum* in the rat, and *T. brucei* and *T. rhodesiense* in the mouse, there is no evidence of any resistance being built up, either toward the rate of reproduction or toward the destruction of the parasites, after they are formed. In two of these infections (rat 619 and rat 703), there is some evidence that the rate of reproduction of the parasites increases, as the infection progresses (rats 703, 705, 709, and 619, and mice 728 and 724).

(*c*) In the relapsing infections: *T. equinum* in the rat, *T. rhodesiense* and *T. brucei* in the dog and guinea pig, the host builds up a resistance directed toward a destruction of the parasites, after they are formed, but it does not affect the rate of reproduction of the remaining parasites (rat 729, dogs 713 and 714, and guinea pigs 719 and 721).

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PROCEEDINGS OF THE SOCIETY OF HYGIENE OF THE SCHOOL OF HYGIENE AND PUBLIC HEALTH OF THE JOHNS HOPKINS UNIVERSITY, BALTIMORE, MARYLAND.

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PHYSICAL EXHAUSTION AND SUSCEPTIBILITY TO PNEUMOCOCCUS INFECTIONS.

By REYNOLD A. SPAETH, Ph.D.,

Department of Physiology, School of Hygiene and Public Health, The Johns Hopkins University.

Miss Oppenheimer and I (American Journal of Hygiene, Volume II, p. 51) have recently shown that in the case of white and hooded rats, physical exhaustion brought about by forced exercise in motor-driven drums decreased markedly the susceptibility of these animals to infections of type I pneumococcus.

This result has now been corroborated in the case of guinea pigs. The experiments have been carried out by Miss Edith Nicholls. Miss Nicholls has found that there is a marked difference in susceptibility to pneumococcus infections between albino and pigmented pigs. This difference is of the order of 300 per cent. The albino pigs are more susceptible. The relation of susceptibility to pneumococcus and extreme fatigue is essentially like that in the case of rats. Animals fatigued three times, after having been injected with a minimal lethal dose of type I pneumococcus, survived in about 50 per cent. of the cases. Fatigue immediately before injection distinctly protected all of the experimental animals, and approximately 83 per cent. remained alive.

In the hope of throwing some light on the nature of the mechanism involved in this protective action of physical exhaustion, we have carried out a series of exploratory experiments with Miss Jean Snyder. Type I pneumococcus has been grown *in vitro* in the heart's blood removed from fatigued animals, and the growth compared with blood removed from resting control animals. We have assumed that there might be some gross inhibiting factor in the blood such as a metabolite which might prevent the growth of type I pneumococcus in fatigued as compared with resting animals. The blood was incubated for varying periods and was then plated out on blood agar and a comparative count was made of the colonies in the two sets of plates.

These experiments have given no indication of the presence of an inhibiting factor detectable by such a gross *in vitro* technique. Pneumococci grew quite as well in the blood from fatigued animals as in the blood of control animals. It seems, therefore, that the inhibiting mechanism may involve the rate of absorption of pneumococci from the peritoneal cavity. Further experiments are now in progress.

THE CLASSIFICATION OF AMERICAN ANOPHELES MOSQUITOES.

By FRANCIS METCALF ROOT, Ph.D.,

Department of Medical Zoology, School of Hygiene and Public Health,
The Johns Hopkins University.

As the result of studies on the wing markings, larvae, and male external genitalia of American *Anopheles* mosquitoes, the classification tabulated below is believed to most nearly represent the probable natural relationships of the various species. The work is still incomplete, studies having been made of the wing markings of only 26 out of the 38 described American species, studies of the larvae in 15 species, and studies of the male genitalia in 19 species. The tabulation given below is tentative, subject to change in minor details when further material, particularly male specimens of some of the South American species, becomes accessible.

One species, *A.* (*Manguinhosia*) *lutzianus,* is not included in the tabulation because no study of it has been made and its position is very uncertain. Other species which have not been studied by the author, but whose position can be indicated with a considerable degree of probability are marked with question signs in the tabulation.

Genus Anopheles.

- Sub-genus *Anopheles.*
  - *Anopheles* group—including *Stethomyia ?, Coelodiazesis, Anopheles, Patagiamyia, Cycloleppteron.*
    - Species—*nimbus ?, barberi, walkeri, atropos, quadrimaculatus, occidentalis, mattogrossensis ?, eiseni, crucians, punctipennis, pseudo-punctipennis, grabhamii, annulipalpis ?.*

Arribalzagia group—including *Arribalzagia.*
Species—*vestitipennis, mediopunctatus, apicimacula, strigimacula, punctimacula, maculipes, pseudomaculipes ?, intermedius ?.*
Sub-genus *Nyssorhynchus.*
Nyssorhynchus group—including *Cellia* (in part).
Species—*argyritarsis, albimanus, tarsimaculatus, braziliensis ?, pictipennis ?, allopha ?.*
Myzorhynchella group—including *Myzorhynchella.*
Species—*lutzii, parvus, nigritarsis ?, gilesi ?.*
Dendropaedium group—including *Dendropaedium, Kerteszia ?.*
Species—*bellator, cruzi, hylephilus, neivai, boliviensis ?.*
Genus CHAGASIA.
Species—*fajardoi.*

THE ACTION OF ULTRAVIOLET LIGHT ON EGG ALBUMIN IN RELATION TO THE ISOELECTRIC POINT.

BY JANET H. CLARK, PH.D..

Department of Physiology, School of Hygiene and Public Health, The Johns Hopkins University.

Ultraviolet light (wave-length less than 300 $\mu\mu$) produces marked physiological effects. Light greater than 300 or 330 $\mu\mu$ produces no effect except in the presence of sensitizers. I have advanced the theory that the action of light on organic substances is initiated by photoelectric action. It is well known that most substances, when illuminated by light of wave-length less than 300 $\mu\mu$, emit electrons so that negatively charged substances are discharged and uncharged substances acquire a positive charge. This is called the photo-electric effect. I have supposed that the first effect of the absorption of ultraviolet light is a discharge of electrons from the light sensitive molecules, this ionized condition leading subsequently to chemical reactions. The ionized condition may be compared to the latent image on a photographic plate. The following experiments with egg albumin give good evidence in favor of this hypothesis.

In a solution of egg albumin the colloidal particles have no charge at the isoelectric point (pH 4.8), a negative charge on the alkaline side of the isoelectric point (pH greater than 4.8), and a positive charge on the acid side (pH less than 4.8).

If the action of light is due to the emission of electrons one would expect the following results on radiating egg albumin with ultraviolet light less than 300 $\mu\mu$. In solutions with pH greater than 4.8 the particles should lose their negatives charges and some would eventually acquire a positive charge, precipitating the others as coagulated albumin, and the result of radiation would be a greater aggregation. For pH = 4.8 the particles being uncharged would, on radiation, become positively charged and repel each other, giving a greater dispersion. For pH less than 4.8 the particles, already positively charged, become more positive and the result is a greater dispersion.

On radiating solutions with pH 4.0, 4.8, 5.4, 6.2, 6.8, 7.2, 7.6, 8.2, these predictions were completely verified. pH 4.0 and 4.8 remain clear on radiation. pH 5.4 precipitates in heavy flakes, the precipitate beginning after fifteen minutes of radiation and being complete after 1 ¼ hours, when the solution is radiated in a quartz test tube at five inches from a quartz mercury arc. pH 6.2 becomes thick and milky, 6.8 faintly opalescent and 7.2, 7.6, etc., remain clear.

Having changed the charge on the colloidal albumin particles one would expect them to show different chemical reactions. Ordinarily at pH = 4.8, or greater than 4.8, egg albumin does not precipitate on half saturation with $(NH_4)_2SO_4$. With pH less than 4.8 it begins to precipitate, the amount of precipitate increasing with decreasing pH. Apparently positively charged albumin particles are precipitated by the bivalent anion SO_4''. When the albumin is negatively charged the precipitation does not take place.

After radiation, therefore, pH 4.0 having a more positive charge should give a larger precipitation with half saturation than before radiation, which it does. Solutions with pH 4.8, 5.4, 6.2, 6.8, 7.2, 7.6, none of which precipitate with half saturation before radiation, all precipitate with half saturation after radiation, the length of time required to give complete precipitation increasing with increasing pH. This all tends to confirm the theory that the first step in light action is an emission of electrons from light sensitive molecules.

One can say therefore that, due to the photo-electric action of ultraviolet light on egg albumin, radiation produces a state of greater aggregation when the albumin particles are negatively charged and a state of greater dispersion when they are uncharged or charged positively. Although other colloidal solutions have not been worked with as yet the principle is undoubtedly of general application. So far no attention has been paid to the importance of the nature of the electrical charge on a substance that is radiated by ultraviolet light and the study of the effect of light on body tissues from this point of view promises interesting results.

SOME OBSERVATIONS ON A CASE OF RAT–BITE FEVER.

By G. H. Robinson, Ph.D.,

Department of Bacteriology, School of Hygiene and Public Health,
The Johns Hopkins University.

A specimen of lymph gland was obtained from a clinical case of rat-bite fever. The tissue was macerated and injected into guinea pigs and white mice. The white mice died, but the guinea pigs did not. An organism could be demonstrated in the blood of the mice which resembled the *Spirochaeta morsusmuris* of Futaki. After seven passages through mice, the organism killed guinea pigs in from two to three weeks. The spirochaetes appeared in a culture medium consisting of 0.2 per cent. infusion agar plus 20 per cent. whole guinea pig blood inoculated with the heart blood of infected animals but could not be found in the second transfer in this medium. The organism conforms to the description of Futaki's spirochaete with the exception that it killed mice regularly in from fourteen to twenty-one days.

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INTEREPIDEMIC INFLUENZA.*

BY EDWIN O. JORDAN.

(Received for publication, February 28, 1922.)

In the years between the great epidemics of 1890 and 1918 the term *influenza* occupied a conspicuous place among the reported causes of death. There were 12,975 deaths reported from this cause in 1917 in the Registration Area of the United States, then containing an estimated population of about 75,000,000. This gives a rate of 172 per million or somewhat more than the rate from either diphtheria (165) or typhoid (134) for the same year. This figure for influenza was not exceptional; the average annual rate from 1906–1910 was 167; in 1916 the rate was 264 and in 1901, 322.† In spite of its relatively high position in official death records, little attention was paid to influenza as a cause of death in the years immediately following the subsidence of the 1890–1893 epidemic. This seems to have been partly because of a conviction that for some time after the great pandemics the term *influenza* has been used to designate forms of illness which prior to the pandemics went by other names. An English observer (Moore, 1907) has given testimony to this effect respecting the years succeeding the pandemic of 1847–48. "When I was a boy the term *influenza* was in constant use to describe an attack of acute catarrh of the respiratory mucous membranes from the nose to the bronchial tubes. It was, as it were, an echo of the great pandemic of influenza, which raged in the winter of 1847–1848. As years rolled by, the name fell more and more into disuse, until *influenza* at last became a mere tradition in medical nomenclature.''

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* From the Department of Hygiene and Bacteriology, The University of Chicago.

This paper represents the essential substance of a De Lamar lecture which was delivered at the School of Hygiene and Public Health of the Johns Hopkins University on January 30, 1922.

† This was the highest rate for the period covered by the available figures (1900–1917).

The view that epidemic influenza is a different disease from what goes by the name *influenza* in ordinary non-epidemic years was clearly set forth by Parsons (1891). This belief was based largely on the difference in mortality in the several age groups, although the clinical divergence between true influenza and severe febrile catarrh was also pointed out. Parsons offers this explanation: ''It seemed probable to me that *influenza* in ordinary times being a term of popular, or loose medical, rather than of strict scientific use, the explanation of the greater number of deaths attributed to this cause in non-epidemic years at the extremes of life might be that the deaths were those of children or old people, with obscure catarrhal or feverish symptoms, which, not being medically attended or at any rate not coming under close medical observation, were ascribed to this conveniently vague cause.''

The loose way in which the term *influenza* has been used has long been the subject of comment by patients as well as by physicians. Humboldt referred to influenza in one of his letters as ''that senseless definition of a pathologic *x*.'' Mrs. Carlyle, who was not in the habit of allowing her ailments to pass unnoted, records at intervals in her letters a number of attacks of what her physician diagnosed as influenza, and on one occasion expresses her scepticism of the current nomenclature: ''Medical men all over the world have merely entered into a tacit agreement to call all sorts of maladies people are liable to, in cold weather, by one name, so that one sort of treatment may serve for all, and their practice be thereby greatly simplified.''

Since 1893 numerous fall and winter epidemics of influenza-like disease have occurred in various parts of the world. By some observers these outbreaks have been apparently regarded as repetitions on a smaller scale of the 1889–90 epidemic, but by others they have been considered to be of a different nature and hence denominated influenza nostras, pseudo-influenza, or grip. Although the French term *la grippe* has been generally used synonymously with influenza, the English term *grip* has been commonly applied with a differential meaning to these winter epidemics and to scattered cases of catarrhal fever. A similar use of differential names was common in Germany. The general public in the United States fastened on the name *grip* and in the years between 1890 and 1918 it was usual to hear from the man in the street who makes his own diagnosis, that he had been suffering from the grip. It is probably true that he sometimes had medical sanction for this nomenclature, and the term *grip* is perhaps as good as any other for an infection or group of infections about which so little is known. What has been commonly called since 1890 an ''attack of the grip'' has seemed in many cases to differ little if at all from what in my boyhood in the seventies and early eighties was called a ''bad cold.'' Deaths from ''grippe'' or ''grip,'' however, have been classed in the official records with the deaths from influenza. Since the 1918 influenza epidemic the term *grip* has been less commonly used. The popular name for all outbreaks of respiratory disease is now ''the flu.''

It is interesting to note the frequency with which the expression ''a type of influenza'' or ''a type of grip'' is used with reference to cases and outbreaks of ill-defined respiratory disease in interepidemic years, as if cautious writers wished to avoid committing themselves irrevocably to the diagnosis of influenza. Thus Adami writes in his War Story of the Canadian Army Medical Corps, ''As might have been expected the illnesses of the men [Salisbury Plain—Winter of 1914–15] were chiefly in connection with the respiratory system. A type of influenza developed, the worst feature being an extremely persistent and troublesome cough. There would be fairly high temperature, pains in the back and limbs and general malaise; but this stage usually passed in three or four days, leaving the cough, which often lasted for weeks. . . .''

While it would hardly be useful to institute any exact comparison between the symptomatology of so protean a disease as epidemic influenza and that of the probably heterogeneous group of ailments catalogued as grip or pseudo-influenza, there are certain more or less familiar differences that may be mentioned here. Broadly speaking epidemic influenza is less often accompanied by sore throat; the mental depression of the patient is often out of all proportion to the other symptoms; cyanosis is more commonly manifest; pneumonia is far more frequent and of a different type. Some cases of grip, however, seem with our present means of differentiation to be clinically indistinguishable from those of epidemic influenza. Stengel (1918) writing of the grip of the years since 1889–90 says ''Taken individually, cases in these so-called 'grip epidemics' could not be distinguished from the milder cases of true influenza observed in the great epidemic, but taken as a whole the recurring winter epidemics did not impress those of us who had seen the great pandemic as being the same disease.''

It is worthy of note that prior to 1890 the designation *influenza* was rare in the health reports of American cities. In Chicago, for example, only 12 deaths are recorded from this cause in the nineteen years 1871–1889. In the State of Massachusetts also very few deaths were reported from influenza between 1842 and 1889 (S. W. Abbott, 1890). In no year were more than 100 deaths recorded, the largest number being in 1857 (92) and the smallest in 1884 (8). Abbott (1890) remarks significantly: ''The average number during the first half of this period was greater than that of the last half, especially when considered with reference to the increase in population.'' In the State of Connecticut there were only 41 deaths from influenza recorded in the ten year period 1876–1885, 3 deaths from this cause in 1886, 4 in 1887, and 9 in 1888. Beginning, however, with the pandemic year 1890, influenza as a reported cause of death becomes much more conspicuous and has never been altogether absent from the death records in most parts of the United States. The ''in-

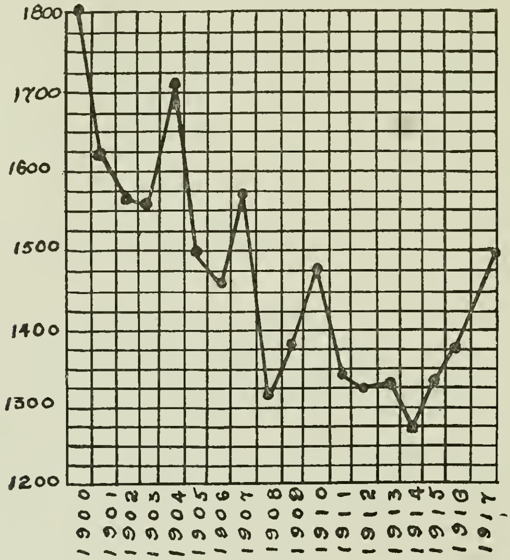

FIG. 1. Pneumonia death rate per million, U. S. Registration Area, 1900–1917 inclusive.

fluenza" death rates in Chicago between 1890–1917 range from 10 per million in 1897 to 1616 in 1916.

It might be anticipated that in general an increase in reported deaths from influenza in the Registration Area of the United States would be accompanied by an increase in reported deaths from pneumonia since deaths from pneumonia are invariably increased in pandemics of influenza, and since the fatal cases of influenza usually if not always terminate in pneumonia. It is seen, however, in Figs. 1 and 2 that along with some marked correspondences in the annual death rates from pneumonia and influenza there have been some notable divergences. The pneumonia rate for 1901 was markedly lower

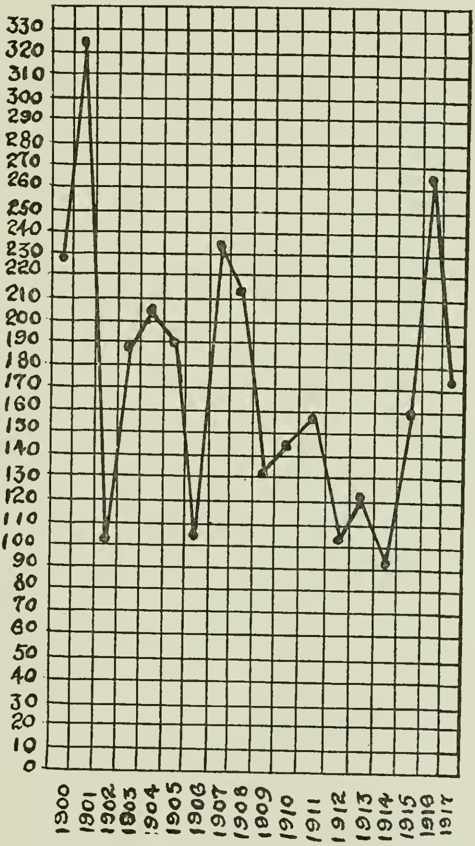

FIG. 2. Influenza death rate per million, U. S. Registration Area, 1900–1917 inclusive.

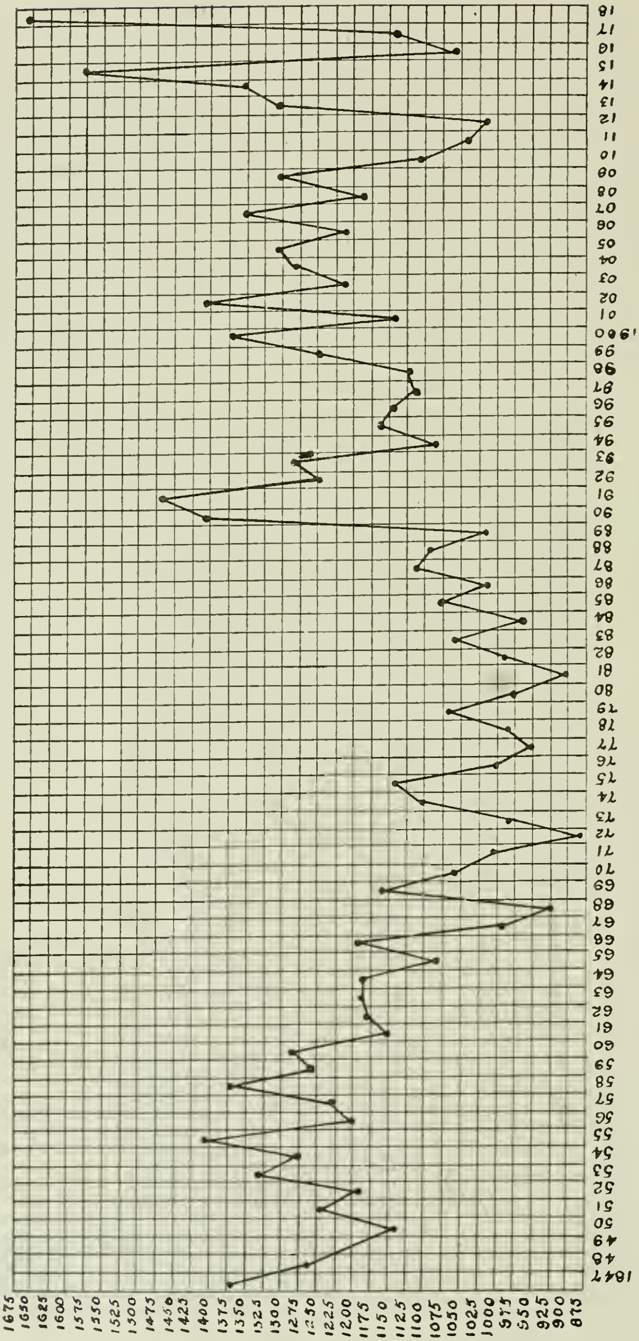

FIG. 3. England and Wales, death rate per million—pneumonia, 1847–1918 inclusive.

than that for 1900 while the influenza rate was much higher in 1901 than in 1900. In 1916–1917 the influenza rate fell while the pneumonia rate rose.

In Chicago in 1896 the pneumonia death rate (including bronchitis) was 2,316 per million and the influenza rate 12; while in 1908, the pneumonia rate was 2,050 and the influenza rate 159. In 1903, the pneumonia rate was very high, but no corresponding increase in the influenza rate was evident. A similar divergence can be observed in the New York City figures as shown for example in the familiar diagrams published by the Prudential Life Insurance Company.

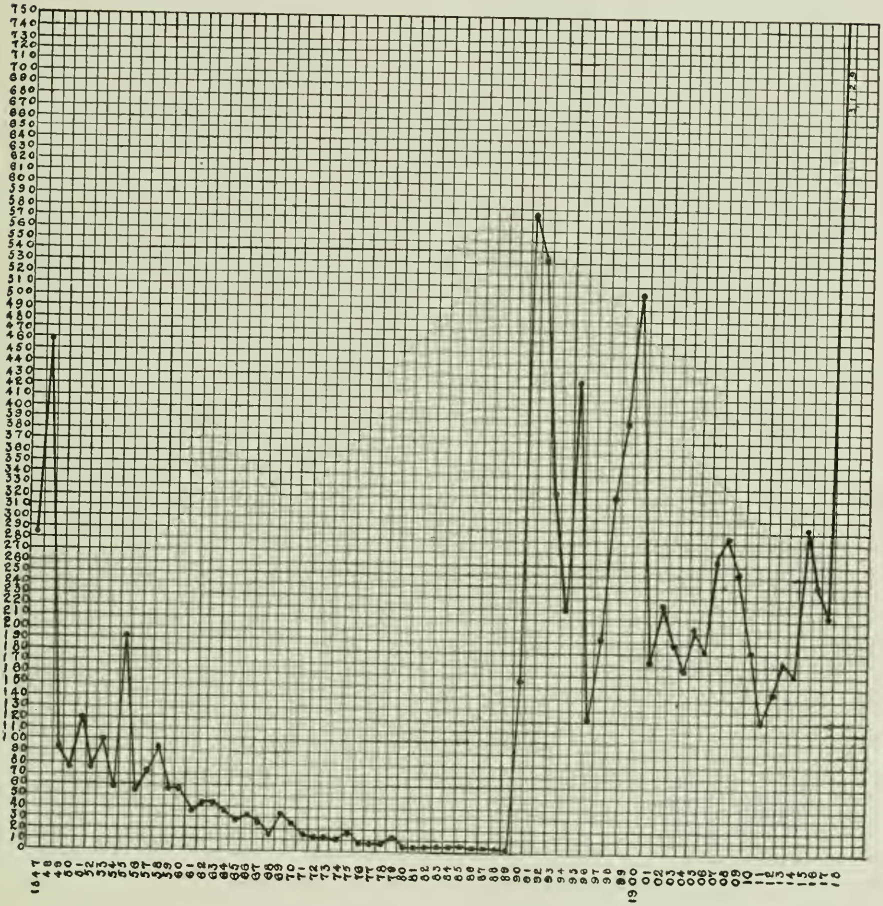

FIG. 4. Death rate from influenza per million inhabitants England and Wales, 1847–1918.

The same lack of concurrence appears in the pneumonia and influenza death rates in England and Wales for the years 1847–1918. The pneumonia death rates for the decade 1850–1860 were very similar to those of 1900–1910, but the influenza rates for these same decades diverge widely. Again while the pneumonia rate for 1902 was slightly higher than that for 1900 the "influenza" rate for 1902 was less than half that for 1900. Other divergences will be observed on inspection of the figures.

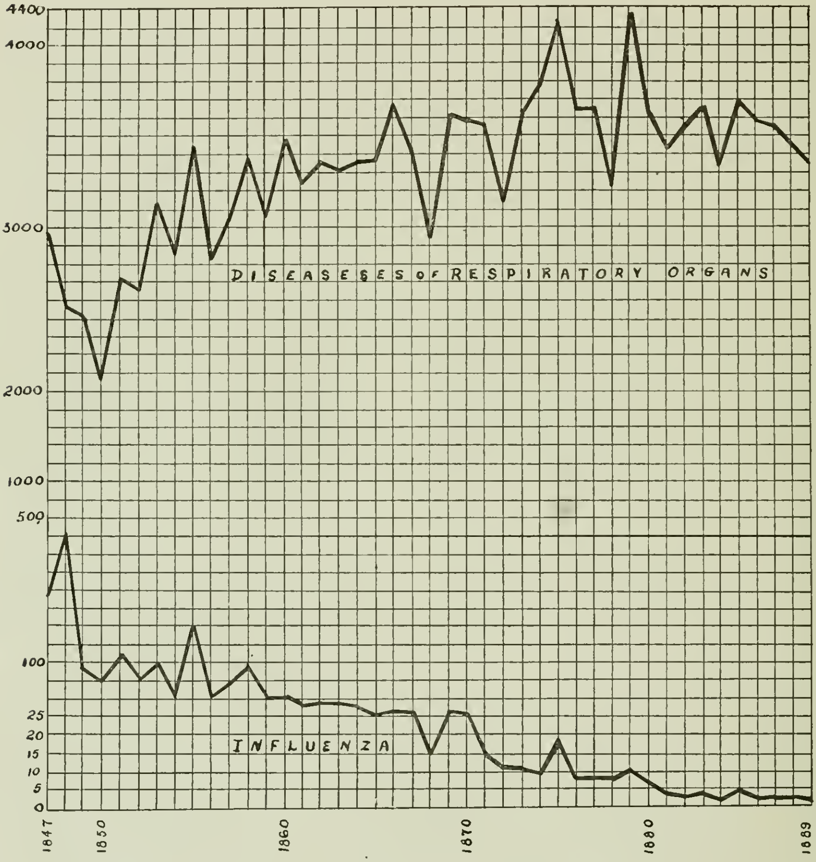

Fig. 5. Death rate per million England and Wales, 1847–1889.

Too much weight, however, should not be attached to a lack of correspondence between pneumonia and influenza waves in interepi-

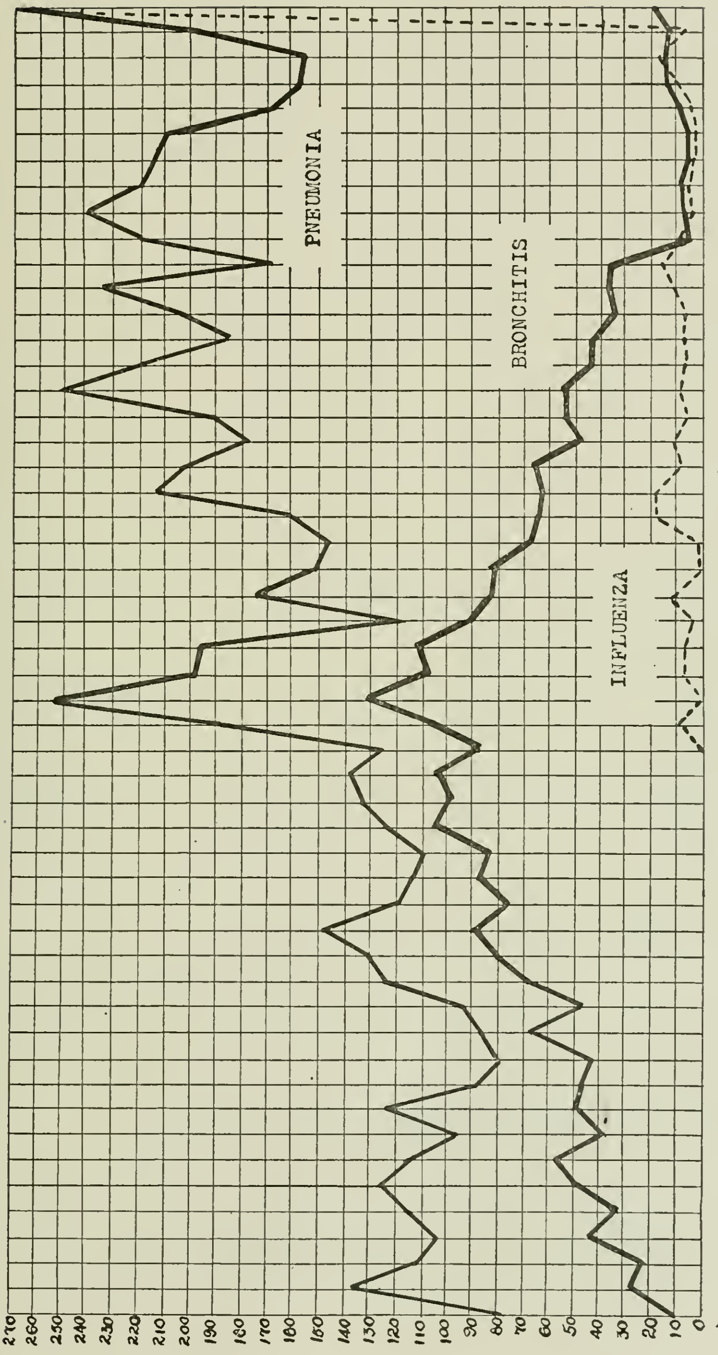

FIG. 6. Chicago, Ill. Death rates per 100,000 from influenza, pneumonia and bronchitis for the period 1867 to 1918 inclusive.

demic years, since the number of deaths from pneumonia is affected by a variety of inconstantly operating factors. The total pneumonia rate in the interepidemic years is so much higher than the influenza rate that fluctuations in the former due to influenza may be swamped by the effect of other causes with a directly opposite tendency. The pneumonia rate without the influenza component is itself a variable.

TABLE I.

Chicago.

Rates per 100,000.

	Pneumonia.	Bronchitis.	Total.
1887–96......	16.70	10.02	26.72
1897–06......	19.38	5.42	24.80
1907–16......	19.83	1.44	21.27

An interesting illustration of shifting nomenclature appears in the deaths recorded under the term *bronchitis.* In Chicago during the last 20 years the trend of reported deaths from this cause has been rather steadily downward.

A similar change has taken place in the figures for bronchitis in the Registration Area of the United States, the rate falling from 45.7 per 100,000 in 1900 to 16.4 in 1917 and 13.3 in 1920.

We may fairly conclude that a large proportion of the deaths formerly reported under the name bronchitis are now reported under the name pneumonia, and that instead of an increase there was probably an actual decrease in the pneumonia rate during the thirty-year period prior to the influenza pandemic of 1918.

That a tendency exists in certain localities to report as grip or influenza deaths that in other localities would be reported as some form of pneumonia or bronchitis is indicated by the influenza rates in Vermont which in the years 1902–1917 were from two to three times as high as the influenza rates for the whole registration area or as those for the neighboring state of Massachusetts.

There are other obvious sources of error in comparing death rates from respiratory disease, but the statistical records at least make clear that in the years between pandemics a certain fatal respiratory disease, or group of respiratory diseases, has been commonly designated by physicians as influenza, that the total mortality recorded under this heading fluctuates considerably, and that since 1890 the name *influenza* has been more commonly used to designate a cause of death than it was before that year.

TABLE II.

Influenza death rates per 100,000.

(From Mortality Statistics, U. S. Census Bureau.)

Year.	Registration Area.	Massachusetts.	Vermont.
1900	22.9	37.1	37.0
1901	32.2	27.7	44.1
1902	10.1	7.6	21.1
1903	18.5	18.9	34.2
1904	20.3	14.9	44.5
1905	19.0	21.6	51.5
1906	10.5	9.8	22.0
1907	24.1	26.3	51.5
1908	22.2	18.9	43.7
1909	13.1	12.9	38.0
1910	14.4	13.2	41.3
1911	15.7	10.0	43.9
1912	10.3	6.9	23.7
1913	12.2	9.2	35.3
1914	9.1	5.9	19.7
1915	16.0	8.6	32.5
1916	26.5	18.5	78.9
1917	17.3	12.6	43.0
1918	302.1	342.0	395.5
1919	98.8	73.9	103.8

In addition to the more or less steady annual occurrence of deaths which have been reported in most civilized countries since 1890 under the name *influenza,* there have occurred a considerable number of more or less generalized outbreaks, also called by observers influenza, which fairly deserve the name of epidemics. A widespread visitation of such a respiratory infection occurred in the early winter of 1900. William James in one of his letters from Southern France to a friend in Cambridge (Jan. 31, 1900) refers to influenza as "devastating Europe." A similar outbreak of a respiratory disease occurred in parts of Europe in the early months of 1905. Italy apparently suffered severely at that time, the Chamber of Deputies having to suspend sittings on account of the illness of many of its members.[1] Vienna also experienced an excessive prevalence of what was seemingly the same respiratory infection. A contemporary report reads: "Influenza is again rife in Vienna. It is as yet of a very mild type, affecting chiefly the alimentary and respiratory organs; but already the occurrence of unpleasant cardiac and ear complications is reported. The hospitals are quite full; on a single day the General Hospital took in 186 new patients, the highest number of admissions recorded for one day for fifty years. The disease chiefly attacks men between 20 and 50 years of age. More than 5,000 cases have been noticed since December 15th, 1904, and doubtless a large number do not come to the notice of the sanitary authorities."[2]

The United States as a whole does not seem to have experienced a high influenza or pneumonia death rate at this time (Figs. 1 and 2). The average influenza death rate in Chicago for 1904–1906 was 66 per million (60, 79, 60) while the average for the decade 1897–1906 was 94 and that for the decade 1907–1916, 90. Similarly the average pneumonia death rate for 1904–1906 was 2,015 per million as compared with 1,958 for 1897–1906 and 1,983 for 1907–1916. The rates for England and Wales (Figs. 4 and 5) were also relatively low for these years. In Massachusetts, however, the month of February, 1905, was marked by an excess mortality from so-called influenza and pneumonia. In Chicago in that month no such excess mortality was manifest. In the United States a more or less characteristic respiratory infection was widespread in the winter of 1915–1916 and was generally regarded as influenza.

Following the subsidence of the 1889–90 pandemic, apparently quite complete in 1896, the years 1898–1901 were marked by an increase in influenza in many places. This rise in turn subsided, little influenza being noted in 1904–1906. In 1907–1908 there was a second wave of respiratory disease designated by the name *influenza*, followed by a longer period of five years, 1910–1914, in which this disease was at very low ebb. The years 1915–1916 witnessed the occurrence of acute respiratory infections in several parts of the world. In the United States these were said by good observers to resemble influenza "in every detail." The following year (1917) infections of this sort were much less prevalent, but this was succeeded by the pandemic years 1918–1920.

[1] Brit. Med. Jour., Feb. 25, 1905, p. 426; March 4, 1905, p. 486.

[2] Brit. Med. Jour., Feb. 4, 1905, 1, p. 273.

TABLE III.

Years of influenza prevalence 1890–1920.

Year.	London.	New York.	Massachusetts.	Chicago.	Connecticut.	New South Wales.	Victoria.
1890....	+	+	+	+	+	+	+
1891....	+	+	+	+	+	+	+
1892....	+	+	+	+	+	±	−
1893....	+	+	+	+	+	−	±
1894....	−	−	+	−	−	+	±
1895....	+	+	+	+	+	+	+
1896....	−	−	−	−	−	±	±
1897....	−	−	+	−	−	±	±
1898....	±	+	−	+	+	+	+
1899....	±	−	+	+	+	+	+
1900....	+	+	+	−	+	±	±
1901....	−	+	+	±	+	+	+
1902....	±	−	−	−	−	−	+
1903....	−	+	−	−	+	±	−
1904....	−	−	−	−	+	±	+
1905....	−	−	+	−	−	−	−
1906	+	−	−	−	−	+	+
1907	−	+	+	+	+	+	+
1908....	+			+	+	−	−
1909....	+	−	−	−	−	−	−
1910....	−	−	−	−	±	−	−
1911....	−	−	−	−	±	−	−
1912....	−	−	−	−	−	−	−
1913....	±	−	−	−	−	−	−
1914....	−	−	−	−	−	−	−
1915....	+	−	+	+	−	−	−
1916	+	+	+	+	+	−	−
1917		−	−	+	+	−	−
1918....	+	+	+	+	+	+	+
1919....	+	+	+	+	+		
1920....	+	+	+	+	+		

In addition to the instances of more or less extensive prevalence in the general population there are on record a number of somewhat sharply localized outbreaks of influenza-like disease, often quite strictly confined to certain communities, sometimes even to institutions. Shera (1917) has described as influenza some 500 cases of infection occurring in South London in the spring of 1915. McNeil and McGowan (1913) recorded an outbreak of ''febrile illness'' in an industrial school near Edinburgh which has been considered (Hamer, 1918) similar to the influenza-like disease in a Glasgow school in 1918 described by McLean (1918). It is very doubtful if all such outbreaks are of the same nature as the true influenza of 1918 and 1889–1890; indeed in some cases the symptoms as described distinctly suggest that they are not. On the whole the localized outbreaks bear much less resemblance to pandemic influenza than do those that are diffused more widely through the population.

It is an important question whether the so-called influenza of interepidemic years (e.g., between 1890 and 1918) is the same disease in whole or in part with the pandemic influenza of 1890 and 1918. Only indirect evidence is available since even now it is impossible to apply valid diagnostic criteria to a case of suspected influenza, and except in pandemic periods there must remain a good deal of doubt as to the etiological identity of reported influenza cases. Until the causal agent or its effects can be recognized, a considerable measure of uncertainty will probably continue to exist.

There are several infectious diseases, such as measles and dengue, that can be quite clearly recognized on the basis of clinical, pathological, and epidemiological features, even though the causal organism has not been identified. Unfortunately, the clinical picture of influenza is not definitely uniform but is generally characterized as ''multiform'' or ''polymorphic.'' Numerous clinical types and varieties of the disease have been described, and the diagnosis of influenza or grippe is often made tentatively or loosely.

It is, therefore, uncertain in what degree the influenza of the years between the great epidemics is identical with the true pandemics of 1890 and 1918. Neither the scattered cases of so-called influenza occurring every winter throughout the world, nor the occasional localized outbreaks of acute respiratory disease in private schools and institutions, nor the wider epidemic diffusion such as took place in parts of Europe in 1905 and more generally through Europe and North America in 1900 can be definitely identified with true pandemic influenza. The outbreak of January–February, 1920, is quite generally, although not universally, regarded as true influenza. The ''flu'' of 1922 shows marked similarity to epidemic influenza, but in some quarters considerable scepticism is expressed as to its identity. There is no doubt in the mind of any competent observer about the strict comparability of the epidemic of 1890 and that of 1918. About all the other so-called influenza of recent years there is more or less difference of opinion. A certain kind of indirect evidence is, however, available.

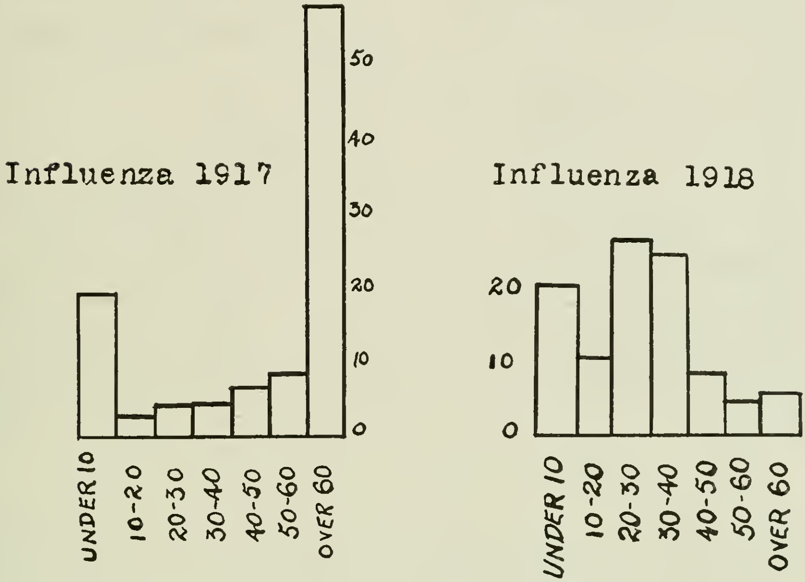

Fig. 7. Age Grouping. Influenza Deaths, U. S. Registration Area.

Age Grouping of Deaths.—During the years 1900 to 1917 more than half of the deaths reported under the name *influenza* were in persons over 60 years of age and less than 10 per cent. in persons between the ages of 20 and 30. In contrast to this, the Census Bureau figures for the Registration Area for 1918 show 22 per cent. of the deaths were in the 20–30 age-group and less than 5 per cent. in the ages above 60.

The reported deaths from pneumonia (all forms) for 1900–1917 and for 1918 manifest a similar disturbance in their age relations. The proportion of deaths in 1918 in the age-group 20–30 is much higher and in the age-group 60 much lower than at any time in the preceding 17 years.

There is ample evidence from English, French, Swedish, and German data that in 1890 the age incidence was substantially the same as in the 1918 pandemic. The insignificant number of deaths attributed to influenza in the United States for several decades before 1890 prevents any direct comparison with those years, but in England and Wales the more common use of the term influenza as a cause of death gives some basis for comparison. The following table from Parsons (1891) (Table IV) shows that from 1876–1889 the larger number of deaths registered in London under the name *influenza* were, like those from pneumonia, among the very young and the very old. It is worth noting also that before 1890 almost one third of the deaths reported under the name influenza were in children under one year of age.

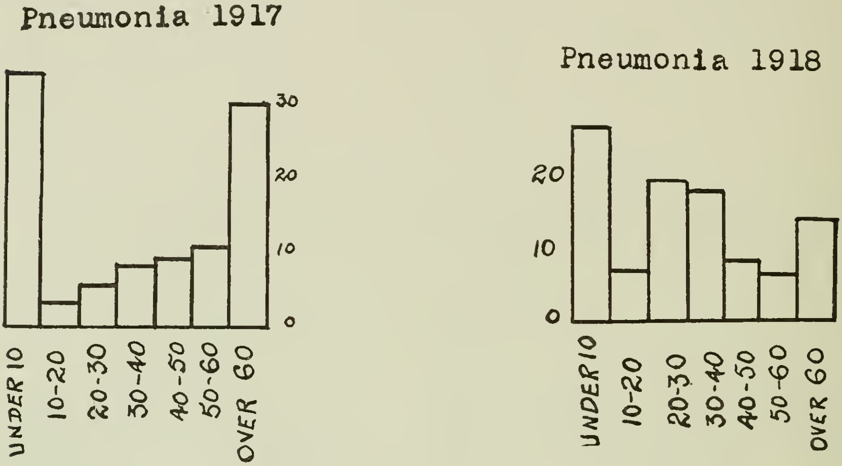

FIG. 8. Age grouping. Pneumonia Deaths, U. S. Registration Area.

In so far, therefore, as age incidence is concerned the epidemic influenza of 1890 and 1918 is rather sharply marked off from the *influenza* of interepidemic years.

TABLE IV.

Proportion at the several ages to 100 deaths from influenza at all ages, in London.

Period.	Percentage at several ages.							
	Under 1.	1–5.	5–20.	20–40.	40–60.	60–80.	80 and above.	Total.
1876–89	32.8	16.0	3.4	3.4	10.0	26.9	7.5	100
First quarter 1890	5.2	4.3	4.7	24.7	36.2	22.4	2.5	100

An interesting special case is afforded by the outbreak of respiratory disease in the United States in December–January, 1915–1916. The deaths from acute respiratory disease for those months were almost doubled in five large cities of the United States as compared with the corresponding months of 1914–1915 (Table V).

The increase in the number of deaths was, however, distributed fairly uniformly over the groups above 20 years of age.

The figures for the three largest cities in the United States (Table VI) present considerable individual differences, but agree in showing that in addition to the marked increase in the 20–40 age, group there was an increase at least equally marked in the ages above 60. The picture presented in these two tables (Tables V and VI) is quite different from the age-grouping of deaths in 1890 and 1918 (Table IV; Fig. 7). While inferences based on mortality alone are obviously unsure it seems fair to conclude that so far as the mortality records go they do not give unmistakable evidence that the respiratory disease prevalent in certain American cities in the winter of 1915–1916 was true epidemic influenza. On the other hand the mortality rates by themselves cannot be said to exclude this hypothesis.

TABLE V.

Pneumonia (all forms), influenza, and bronchitis in Baltimore, Chicago, New York, Philadelphia, and Providence.

Ages.	December–January, 1914–1915.	December–January, 1915–1916.	Per cent. excess.
0–20	2,050	2,418	17.9
20–40	409	1,071	161.9
40–60	788	1,856	135.5
60	1,022	2,753	168.4
Total	4,269	8,098	
	Per cent.	Per cent.	
0–20	48.0	29.9	
20–40	9.6	13.2	
40–60	18.4	22.9	
60	24.0	34.0	

Racial Mortality.—A further distinction between the 1918 pandemic and the influenza of the preceding years lies in the different

TABLE VI.

Deaths from pneumonia (all forms), bronchitis, and influenza.

Per cent. excess in December–January, 1915–16, over December–January, 1914–15.

Ages.	New York.	Chicago.	Philadelphia.
0–20	−1.1	+47.7	+63.3
20–40	+136.2	+192.0	+195.7
40–60	+100.0	+209.6	+168.2
60+	+114.2	+318.7	+239.0

racial mortality. The 1918 epidemic was characterized in the United States by less increase in influenza mortality among the colored race than among the white although the former usually suffer most severely from respiratory diseases. The facts are available for several registration states with considerable white and colored populations (Mortality Statistics, 1918, U. S. Census Bureau).

TABLE VII.

Ratio of influenza and pneumonia deaths in colored population to those in white population.

State.	Influenza.		Pneumonia (all forms).	
	1911–1917.	1918.	1911–1917.	1918.
Kentucky	1.71	1.01	2.02	2.14
Maryland	1.77	1.16	2.35	1.59
North Carolina	1.43	1.44	2.40	1.79
South Carolina	1.44*	1.49	1.48*	1.11
Virginia	1.29†	0.95	2.10†	1.93

In every instance there is less racial difference in 1918 between either the influenza or the pneumonia rate or both than in 1911–1917. This can only be interpreted as indicating that the white race is more susceptible or the colored less so to the pandemic influenza of 1918 than to the influenza of the years immediately preceding.

* 1916–1917 only.

† 1913–1917 only.

The same condition is shown in another way and yet more clearly by a comparison of the white and colored deaths from influenza and pneumonia in the last four months of 1918 with those for the corresponding period in 1915 (Table VIII).

TABLE VIII.

Number of times deaths from influenza and pneumonia (all forms) during the last four months of 1918 are of corresponding deaths in 1915.
(Taken from Column 6, p. 28, Mortality Statistics, 1918, U. S. Bureau of Census.)

State.	White.	Colored.
Kentucky	14.4	7.0
Maryland	15.8	7.9
Virginia	18.3	9.3
City.		
Birmingham	14.8	11.1
Atlanta	10.8	5.6
Louisville	17.6	4.7
Washington, D. C.	16.3	6.9
New Orleans	9.8	5.2
Baltimore	13.6	6.2
Memphis	13.2	4.6
Nashville	17.1	5.8
Richmond	14.2	5.6

It is evident that the greater relative increase in influenza-pneumonia mortality in the white race as compared with the negro constitutes a distinguishing feature of the 1918 epidemic and serves to mark it off from the influenza of the pre-epidemic years.

Mortality according to Sex Grouping.—It has been pointed out by Craig and Dublin (1920) that there is a sex difference as well as an age and race difference between the deaths attributed to influenza in 1910–1917 and those in 1918. In the first period the female death rate in both white and colored races was uniformly higher than the male. In 1918 the white male mortality was higher than the female, and while the colored female mortality was still somewhat higher than the male the excess female rate is much less for 1918 than for 1911–1916.

The 1918 influenza epidemic is, therefore, sharply marked off from the influenza of the preceding years by (1) the greater relative mortality at ages 25–35; (2) the relatively increased mortality in the white race as compared with the negro; (3) the higher mortality in white males than in white females; (4) the relatively increased mortality in colored males as compared with females of the same race.

The lack of correspondence between influenza and pneumonia rates already mentioned is more difficult to evaluate and need not be stressed here. The absence of radiation or dispersiveness in the interepidemic outbreaks of influenza-like disease is a feature that is as striking as it is imperfectly understood. All the great recognized pandemics of influenza have been characterized by a high degree of infectivity or dispersiveness. Daniel Drake in 1807 expressed his amazement at the way true epidemic influenza penetrated the remote settlements of the Middle West in spite of the poor means of communication.

Although the doctrine of changes in type of disease has sometimes been advanced as an explanation of the difference between the 1918 influenza and the so-called influenza of the preceding decade, fundamental differences in age, sex, and race mortality in different outbreaks of a specific disease have never been shown to occur, and are difficult to explain as epidemic manifestations recurring uniformly at irregular intervals.

There is no escape from the conclusion that the so-called influenza of interepidemic years is in whole or in part different from the influenza of the great epidemics of 1918 and 1890. A great many of the deaths recorded under the name influenza in the years between epidemics are certainly not due to the same microbic causes as those occurring in epidemic years. Is this true of all? Eliminating the deaths attributed to interepidemic influenza on grounds of clinical similarity, local and temporary fashions in medical nomenclature, and other unessential reasons, is there still in Europe and North America a residue of cases and deaths from true influenza which keeps a thread of continuity between the great outbreaks? This question it is not now possible to answer, and perhaps no answer will be possible until the causal agent can be surely and readily identified. Meanwhile localized outbreaks and indeed individual cases of influenza-like disease and of all mild respiratory diseases seem to demand more searching study than they have yet received, bacteriologically, epidemiologically and clinically.

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THE ANTHRAX HAZARD IN PENNSYLVANIA TANNERIES.*

BY HENRY FIELD SMYTH.

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During the latter half of the year 1921, the Pennsylvania State Department of Labor authorized a survey of the entire tanning industry in the State to determine the anthrax hazard of the industry in its various branches and the incidence of human anthrax within the State, traceable to the handling of hides and skins. The author of this paper carried out all the laboratory tests connected with the survey, collecting practically all of the samples in person, and coöperated with Dr. Elizabeth Bricker, Medical Inspector of the department in the field surveys. A summary of this survey including the former study of anthrax in the horsehair industry is appearing elsewhere under the title of "Industrial Anthrax in Pennsylvania" (1922). It is the object of this paper to report in more detail the laboratory work carried out and to discuss some of the results thereof.

Practically every tannery in Pennsylvania that was operating at the time was visited and whenever raw or untanned stock was on hand, samples were taken for laboratory examination. In our statistical studies were included all tanneries visited and all tanneries closed at the time but which had been responsible for cases of anthrax in past years. This included in our statistical studies almost every cattle hide and goat skin tannery in the State. In addition to the tanning of cattle hides for sole leather, upper leather, bag, strap, belt, and sport and enamel leather, and the tanning of goat skins for glazed kid and enamel leather, there is a little domestic horse and colt hide tanned for sport leather and enameling and some tanning of sheep skins for fancy colored leathers and for chamois. A few kangaroo skins are tanned for glazed kid and a few water buffalo hides for sole leather, but these are included in the work of goat skin and cattle hide tanning respectively and are not considered separately. Table I shows the number of tanneries handling the different kinds of raw material at the time of the survey. As we have found no anthrax in domestic hides or skins in this State and as no imported horse hides were found, no detailed studies will be reported from tanneries handling horse hide. Sheep skin tanneries also will be omitted from the study as all imported sheep skins at present reach the tanneries in a salt and sulphuric acid pickle. No anthrax is likely to be found in these skins and no anthrax cases are on record in this State traceable to them.

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* From the Department of Industrial Hygiene, School of Hygiene, University of Pennsylvania.

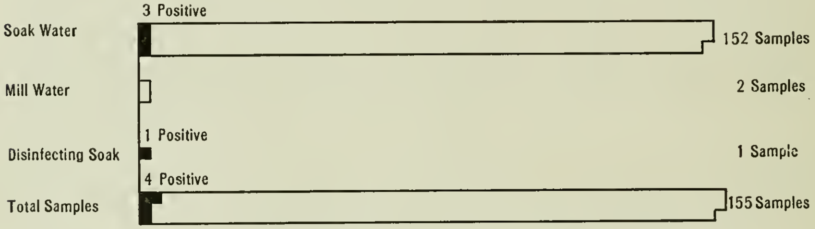
This narrows the scope of this report to a consideration of conditions connected with the bark and extract tanning of cattle and buffalo hides and the chrome tanning of goat skins. Forty-seven cattle hide tanneries were visited, and samples were taken from all but 3 of them. Sixteen goat skin tanneries were visited, and samples were taken from 11. Two were closed down and 3 had no raw or unlimed stock on hand. Table I shows the number and types of tanneries visited. Laboratory tests to be reported below show that at least most of the anthrax risk is in the handling of dry or of limed hides or skins, so the tanning processes after dehairing have little bearing on our problem and will therefore not be discussed here.

TABLE I.

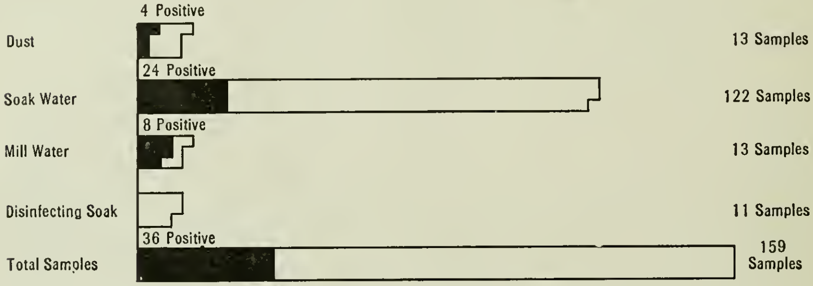
Kind of hides being tanned.

47 tanneries visited were tanning Cattle Hides
2 tanneries visited were tanning Horse Hides
2 tanneries visited were tanning Buffalo Hides
3 tanneries visited were tanning Calf Skins
16 tanneries visited were tanning Goat Skins
6 tanneries visited were tanning Sheep Skins
1 tannery visited was tanning Kangaroo Skins.
2 factories visited were enameling Colt, Kid and Kip Skins.

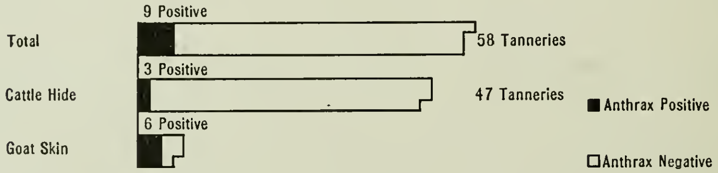
So far, domestic hides from Pennsylvania do not seem to have been responsible for the introduction of anthrax into the tanning industry in this State, and we have recovered no anthrax cultures from domestic hides in the 11 tanneries handling them. However, we have previously tabulated 17 cases of anthrax in Pennsylvania from 1910 to 1919 that were not directly traceable to any industry (1921), so we feel that the time may come when anthrax will be a menace in domestic cattle hides. We have a history of one case of anthrax contracted from handling Texas hides. Over 50 per cent. of the cattle hides tanned in the State are from domestic packer-killed cattle. A small percentage as stated above comes from local slaughter houses. Among imported cattle hides a few come from the West Indies, and a few from China, Mexico, and India, from any one of which three countries anthrax may be imported. We have records of anthrax cases traceable to handling of cattle hides from all of them. The bulk of the imported cattle hides, however, come from South America; many, but not all of them, coming from packer-killed cattle. These packer hides are now shipped in the so-called green salted condition, still more or less moist with brine and tied individually in square bundles with the hair side in. They give rise to no dust in handling and we have no record of any anthrax cases traceable with certainty



Cattle hide tanneries.



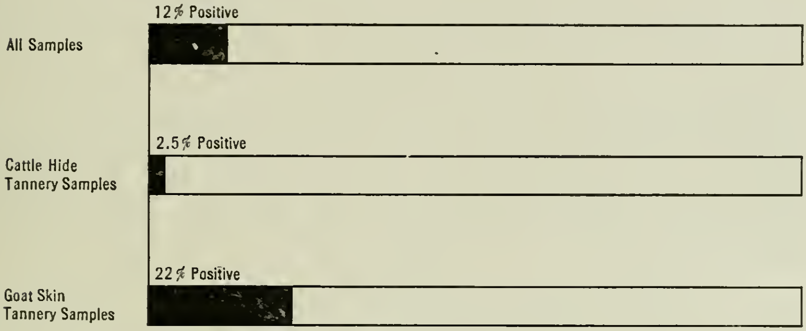
Goat skin tanneries.



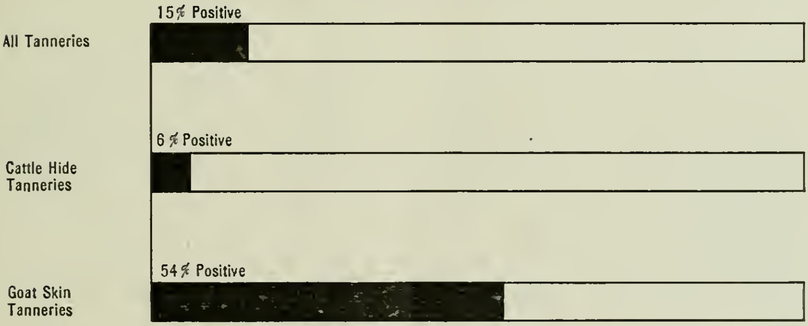
Tanneries in which anthrax was found.

CHART I. Tannery samples cultured for anthrax.

to packer hides. It is not at all likely that beef cattle would be killed for meat when anthrax infested. Some of the South American hides, however, are not from packer-killed cattle, and these are sometimes shipped dry or dry salted. We have records of anthrax contracted from these South American hides and have isolated virulent anthrax from vats in which such green salted non-packer hides were soaking. (See Table V.)



Examples examined.



Tanneries sampled.

CHART II. Percentage of anthrax cultures obtained.

Before they are ready for tanning, hides must be softened up and dehaired. If dry or if very dirty, cattle hides are placed in tumbling mills with water to soften and clean. If green salted this is not done except with dirty hides. The hides are then tied end to end in long ropes and placed to soak in large vats. Soaking practices differ somewhat; some tanneries soaking in still water and some in running water and still others in a weak sulphide solution. Hides soak from 4 to 24 hours in a vat and are then transferred to another vat over reels with little or no direct handling. Soaking continues from one to four days, usually passing through at least four soak vats. Then hides are reeled into 20 per cent., or stronger, lime suspension with or without sulphide to prepare for dehairing. Lime soaking continues from 4 to 8 days or more, the hides being reeled through several vats during this time. They are not handled individually until ready for dehairing. The strong limes kill most of the anthrax spores and practically all of the viable ones remaining are removed in the dehairing and subsequent washing so that we consider that the direct exposure to anthrax hazard ceases when the hides are ready to tan. As will be shown below, our records of anthrax cases and our laboratory tests indicate that this is so. The occasional cases of anthrax contracted in the finishing departments are probably indirect dust or contact infections from the untanned hide department.

TABLE II.

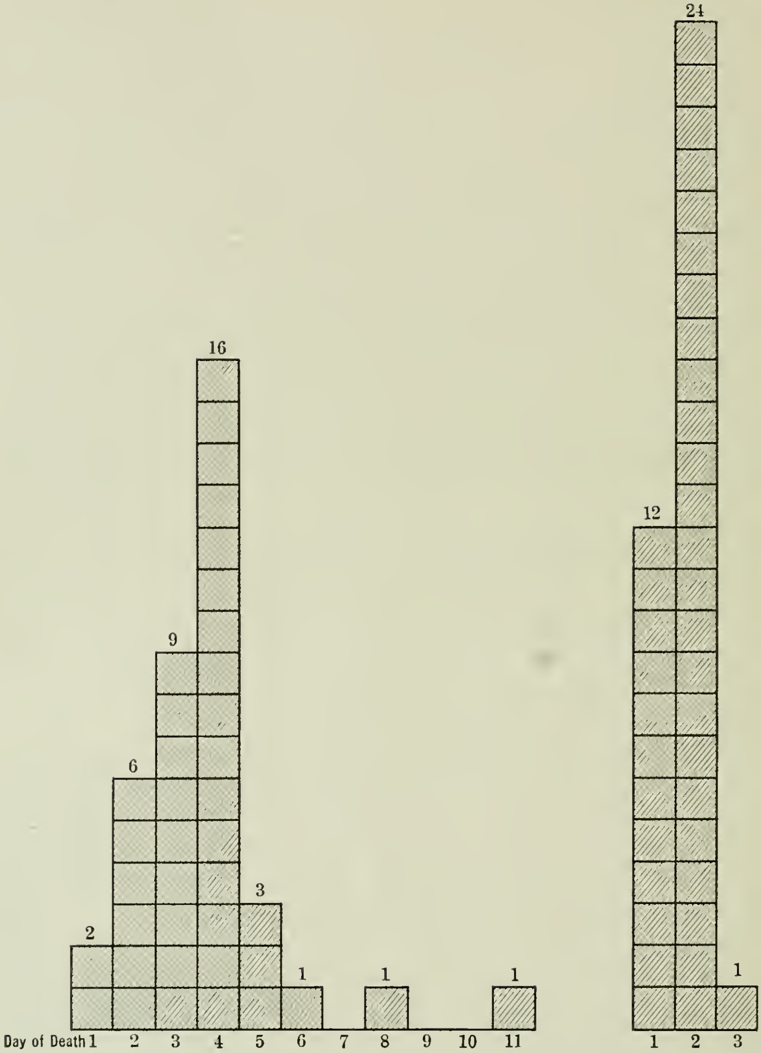
Sources of hides and skins tanned in Pennsylvania.

Country.	Tanneries Receiving.			
	Cattle Hides.	Horse Hides.	Goat Skins.	Sheep Skins.
U. S. A.	47	3	1	3
South America	32		4	2
West Indies	1		2	
Canada	1			
Mexico	3		1	
India	5		11	
China	1		6	
Europe			5	1
Australia			3	
Java			5	
Arabia			2	
Africa			1	
Russia			1	
New Zealand				1

Goat skins in Pennsylvania are practically all imported, mostly from countries where anthrax is more or less prevalent. (See Table II.) Only one of the 16 glazed kid tanneries visited was receiving any domestic skins and that one was tanning mostly imported ones. Some of these skins are imported in the wet salt state in casks and so are not apt to infect the freight handler, but the majority of them come into the country dry and packed in large close bales, covered with burlap or woven split-bamboo or other covers. There is much dust from the handling of these bales and we have records of several freight handlers infected therefrom. Before tanning, these bales are opened up and the skins inspected and weighed by hand. The skins are then placed to soak in plain water for from 24 to 48 hours. They soak continuously in the same vats without change of water. Skins are handled individually and the dried skins with their irregular and often sharp edges offer opportunities for skin traumatism and possible resultant anthrax infection. The dust and sweepings of stock rooms where these dry skins are stored frequently harbor anthrax spores. At the end of the soaking period vats are drained and the skins pulled out by hand and thrown into tumbling mills where they are washed in running water for a short time. The drippings from these mills and the water on the floor under them are very apt to contain anthrax spores. In emptying vats the men have to get into the vat on the wet skins and their clothing becomes wet with water which may contain anthrax. After the washing the mill contents are dumped on the floor and the skins taken to the beam house where they are trimmed over a beam and, if need be, slit open and then placed in the dehairing lime vats, where they remain for several days. Sometimes they are changed from vat to vat in the limes and this requires additional handling. The limes may contain suphide or arsenic to aid in the depilatory action. From the limes the skins go to the dehairing machines. Beyond this point we feel that, as previously stated, there is little risk of anthrax infection.

United States Government regulations, as promulgated by the Bureau of Animal Industry (1918), state that all imported hides and skins must be subjected to disinfection unless coming in with a Consular certificate, stating that the district from which they were shipped was free from anthrax. Osborn (1920), Seymour-Jones (1922), the author, and others have pointed out the futility of this provision and the fact is substantiated, as will be seen by the isolation of virulent anthrax cultures from such certified material.

At the beginning of this investigation samples were taken from dry or wet salted skins, from dust of stock rooms, from plain water



Animals injected with tannery samples. Animals injected with pure culture.

Chart III. Time of death of guinea pigs inoculated with anthrax.

soaks and mill drippings, and from disinfecting and dehairing soaks. Skin samples consisted of bits of skin several square inches in area cut from the edges of three skins from each bale sampled. At the laboratory pieces about one fourth to one half square inch in area were cut from these for culture. Dust and liquid samples were collected in sterile, numbered, rubber stoppered test tubes. Vat samples were dipped from the top of vats in which skins were soaking and mill water samples were taken directly from mill drippings or from streams of water on the floor beneath the mills. Average samples indicative of average hazards were aimed at and not samples representing maximum risks. Samples of vat sludge would, we feel sure, have given more positive cultures but the workers are more exposed to surface splashings and least to the sludge which is hosed out before the vat is scrubbed.

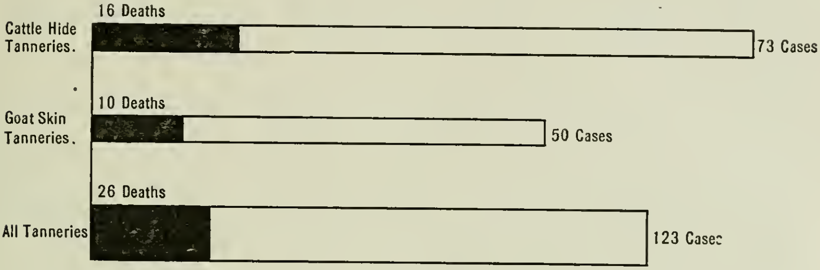


CHART IV. Anthrax cases and deaths in Pennsylvania Tanneries, 1910–1921.

Dry samples, skin or dust, were covered with bouillon or dextrose-serum bouillon and incubated for 48 hours or more. Agar plates were then poured therefrom, after heating to 70° C. to destroy non-spore formers, and typical curled anthrax surface colonies as described by Hagan (1920) or deep colonies as illustrated by Leake and Lederer (1919) were searched for. When found they were planted on agar and incubated. Twenty-four-hour growths of pure cultures were suspended in physiological salt solution and injected into guinea pigs. Liquid samples that might contain substances harmful to guinea pigs (as disinfectant and dehairing soaks) were inoculated into bouillon or dextrose-serum bouillon and plated as above. Plain water soaks and mill liquors were heated to 70° C. or over for 10 minutes or more and then one or several cubic centimeters therefrom were injected subcutaneously over the abdomen of numbered guinea pigs. Feeling that the use of dextrose-serum bouillon might yield positive cultures from non-virulent organisms (1921) its use was not long continued, for as stated above, the demonstration of average, rather than maximum, risks was aimed at.

Inoculated guinea pigs were kept under observation for two weeks and any dying within that time were autopsied. Smears were stained and agar slants were made from the spleen pulp and the heart blood regardless of whether rods were or were not found subcutaneously and regardless of the character of the gross lesions. When typical anthrax-like organisms were isolated from the heart-blood agar slant, cultures of the same were suspended in physiological saline and injected subcutaneously into other guinea pigs. When the second pig injected with pure culture died with typical gelatinous oedema spreading from the inoculation site, with a soft dark pulpy spleen and with square-ended gram-positive rods in large numbers in the blood and spleen, the diagnosis of anthrax was made. This confirmatory inoculation of pure cultures was carried out in all but a few repeat tests in one glazed kid factory, where it was omitted on account of a shortage of animals. We feel that though we might have obtained more positive cultures by the use of mice or rats instead of pigs, yet some of these might not have been truly virulent and this might have indicated a higher incidence of anthrax hazard than was really present. We feel confident that by this method we obtained no false positives.

Sixty-seven goat skin samples were taken, 15 of them from uncertified, undisinfected skins, but no typical anthrax cultures were obtained from them. A number of suspicious cultures failed to kill the second pig and so were declared negative. This method of sampling was then abandoned as unlikely to yield positive results. One or two infected skins will contaminate an entire vat of soaking skins, but would probably be overlooked in sampling. It would hardly be likely that an entire bale would be infected and the skins from known diseased animals would be likely to be secreted in the center of the bale. Nine samples from dehairing lime soaks were cultured but all proved negative, and as laboratory tests showed that the strong lime suspensions rapidly reduced the numbers of anthrax spores, no samples were taken. Twelve samples from disinfecting lime soaks (10 per cent.) also failed to yield anthrax cultures.

In all, 40 virulent anthrax cultures were isolated from 314 samples of dust or soaks collected in 64 tanneries, 9 of which gave positives. This makes 12 per cent. of the cultures positive and 14 per cent. of the tanneries positive for anthrax. (See Table III and Chart I.) Of the 39 guinea pigs yielding anthrax-positive cultures when inoculated with samples or cultures from samples, 2 died in 1 day, 6 in 2

TABLE III.

Anthrax cultures obtained from tannery samples.

Samples.	Number of samples taken and of anthrax cultures isolated.								
	Total samples.	Anthrax cultured.		Cattle hide tanneries.			Goat skin tanneries.		
		No.	Per cent.	No. samples.	Anthrax cultured.		No. samples.	Anthrax cultured.	
					No.	Per cent.		No.	Per cent.
Dust	13	4	30	0	0	0	13	4	30
Plain soak	274	27	9.8	152	3	2	122	24	19.6
Mill water	15	8	53.3	2	0	0	13	8	61
Disinfecting soak	12	1	8.3	1	1	100	11	0	0
Total	314	40	12−	155	4	2.5−	159	36	22−

Tanneries in which anthrax was found.	Tanneries sampled.	No. yielding anthrax.	Per cent.	Cattle hide tanneries.			Goat skin tanneries.		
				Tanneries sampled.	Yielding anthrax.	Per cent.	Tanneries sampled.	Yielding anthrax.	Per cent.
	64	9	14−	47	3	6	11	6	54−

days, 9 in 3 days, 16 in 4 days, 3 in 5 days, 1 in 6 days, 1 in 8 days, and 1 in 11 days from the date of inoculation, giving an average of a little under 4 days, but indicating that an inoculated animal should be kept under observation for at least 11 or 12 days before being pronounced negative. Thirty-seven guinea pigs died from the injection of pure cultures of anthrax isolated from other guinea pigs or from dust samples. Of these 12 died within 24 hours, 24 within 2 days, and 1 on the third day. When pure cultures are injected, probably one week is ample time for observation. (See Table IV and Chart III.)

TABLE IV.

Time of Death of Guinea Pigs with anthrax.

	Pigs injected with mixed cultures.	Pigs injected with pure cultures.
Died in	No. animals	No. animals
1 day	2 animals	12 animals
2 days	6 animals	24 animals
3 days	9 animals	1 animal
4 days	16 animals	
5 days	3 animals	
6 days	1 animal	
8 days	1 animal	
11 days	1 animal	
Total	39 animals	37 animals

In the laboratory examination of field samples the following positive results were obtained: (See Table V.) From cattle hide tanneries four virulent anthrax cultures were isolated. Tannery No. 6 was soaking only green salted South American certified hides at the time (Sept. 28). Samples from two plain soak vats gave anthrax positives. They had received no dry hides since July and the dry hides received at that time were certified. The anthrax may have come from those dry hides, either remaining in the vat from a former soak or contaminating the green salted hides as dust from the stock room. Tannery No. 12 was soaking uncertified India water-buffalo hides in a 1–1,000 mercuric chloride solution in which they had remained for 24 hours. This is the usual method of disinfection employed by the cattle hide tanners. A sample from this vat was heated and injected into a guinea pig and at the same time was plated out on agar. Curled colonies forming on agar were cultured and injected into a second pig. Both guinea pigs died of virulent anthrax. Tannery No. 24 was soaking certified South American green salted hides in a weak sulphide solution. This tannery uses non-packer hides but does not receive any that are not certified. One vat sample yielded anthrax. This showed four anthrax cultures, three from certified hides. One of these was surely from green salted hides, but the other two may have been from dry hides. One culture was from a so-called disinfectant soak as permitted by the Bureau of Animal Industry regulations.

Anthrax was isolated from six out of eleven goat-skin tanneries visited, as follows: Tannery *G* was visited a number of times for the

TABLE V.

Anthrax isolated from tannery samples.

Tannery.	Material tanned.	Sample.		Source of raw stock.	Condition of raw stock.	Certified stock.
		No.	Kind.			
6.....	Cattle hides	209	Soak water	S. America	Green salt	Yes
		210	" "	" "	" "	Yes
12....	Buffalo hides	229	$HgCl_2$ 1–1000	India	Dry	No
24....	Cattle hides	279	Sulphide soak	S. America	Green salt	Yes
G....	Goat skin	2	Soak water	Imported	Dry	?
		10	Mill water	"	"	?
		11	" "	"	"	?
D....	" "	72	Soak water	Arabia	"	Yes
		78	Mill water	"	"	Yes
A....	" "	87	" "	Imported	"	?
		92	" "	"	"	?
H....	" "	290	Dust	"	"	Yes
I.....	" "	295	Soak water	"	"	Yes
		297	Mill water	"	"	Yes
		298	" "	"	"	Yes
		300	Dust	"	"	Yes
A....	" "	308	Soak water	"	"	?
		310	" "	"	"	?
		313	" "	"	"	?
		314	" "	"	"	?
		316	" "	"	"	?
		317	" "	"	"	?
		320	" "	"	"	?
J.....	" "	323	Dust	"	"	Yes
		324	"	"	"	Yes
		326	Mill water	"	"	Yes
G....	" "	328	Soak water	"	"	?
		331	" "	"	"	?
		333	" "	"	"	?
		334	" "	"	"	?
		336	" "	"	"	?
		338	" "	"	"	?
		344	" "	"	"	?
		352	" "	"	"	?
		362	" "	"	"	?
A....	" "	375	" "	"	"	Yes
G....	" "	378	" "	"	"	?
		382	" "	"	"	?
		383	" "	"	"	?
		385	" "	"	"	?

purpose of making detailed studies to determine if it was possible to demonstrate the seeding of a vat with anthrax so that every batch of skins soaked therein would become infected. From this tannery there were cultured 2 mill water samples, both positive for anthrax, and 74 soak water samples (from 26 vats), 14 of which yielded anthrax. Four vats were cultured as many as ten times. In two instances anthrax was isolated three times from the same vat, but never oftener than three times in succession. Most of the glazed kid tanneries handle both certified and uncertified skins and where no disinfection process is used we were not always able to tell what kind of skins were soaking in the vats sampled, or even the country from which they came. The Bureau of Animal Industry regulations do not require any skin disinfection, provided the waste soak liquors are treated with 250 parts per million of chlorine gas before discharge into sewage. This tannery is the only one that takes advantage of this provision, which of course offers no protection to the tannery worker. Tannery *D* was handling both certified and uncertified skins, the latter being soaked in a 10 per cent. lime suspension. Five samples from certified stock gave two anthrax cultures, one from soak water and one from mill water. One sample from the disinfecting lime solution was negative. Tannery A was visited several times. On the first visit two anthrax-positive samples were taken from mills. Later serial samples were taken from 4 vats, *A, B, C,* and *D,* sampling every 48 hours, at the end of a soaking period. Of these 16 samples, 7 yielded anthrax, vat *B* giving three positives out of four samples. This vat was then drained and thoroughly whitewashed and left idle for 24 hours. It was then put in service again and a second series of samples taken. The first three, on the second, fourth, and sixth day after whitewashing were negative. The fourth on the eighth day was positive, but this may have been due to a reseeding of the vat from fresh infected skins. The skins soaking when the sixth day sample was taken had been disinfected previously, but may still have contained viable spores. Tannery *H* gave no positive cultures from vats or mills, but the dust from the stock room where none but certified skins were stored gave anthrax. Tannery *I* handles South American skins, mostly certified. Here six soak and mill water samples gave three anthrax positives and anthrax was isolated from one dust sample. Tannery *J* gave three positive anthrax cultures, two from dust samples and one from mill water. This tannery had received but one shipment of uncertified skins within the year and that one was received over two months before the samples were taken.

In goat skin tanneries 122 soak water samples gave 24 anthrax cultures, or 19.6 per cent. positives. Thirteen mill water samples gave 8 anthrax cultures or 61 per cent. positives. Thirteen dust samples gave 4 anthrax cultures, or 30 per cent. positives. This gave 36 positive anthrax cultures in 159 samples, over 22 per cent. The positives were obtained in 6 of the 11 tanneries sampled, or over 54 per cent., showing that at the time of the survey there were virulent anthrax bacilli in over one half of the goat skin tanneries visited. Of the 36 samples yielding anthrax, at least 14 were from certified, supposedly anthrax-free skins, and probably most of the others also.

As no anthrax organisms were isolated from any of the lime soaks sampled, a series of tests were planned to determine the probable efficiency of calcium oxide disinfection. The Bureau of Animal Industry regulations formerly called for a 12-hour soak in a 5 per cent. suspension of CaO, but recently they have modified this provision and permit "an immersion for not less that 12 hours in a lime suspension made by adding 15 pounds of quick lime or 15 pounds of hydrated lime to each hundred gallons of water." The chief of the Bureau states in a personal communication to Dr. Bricker that "this gives an approximate equivalent of 5 per cent. of calcium oxide." Commercial limes are never 100 per cent. CaO and 75 per cent. is a fair average, though some may run as low as 50 per cent. With 50 per cent. lime the above proportion would give a 0.89 per cent. suspension, with 75 per cent. lime a 1.3 per cent., and with 100 per cent. lime a 1.7 suspension, a rather poor equivalent for 5 per cent. To give a fair test of the efficiency of limes the first test used suspensions of both rock lime and of hydrated lime. Small bits of skin one fourth to one half square inch in area were sterilized in the autoclave, and then covered with a suspension of anthrax spores and drained. They were then immersed in the lime suspension in covered beakers at room temperature. At stated intervals the limes were well stirred and bits of skin removed, washed in sterile water, and cultured in bouillon.

The first series used 1.8 per cent., 5 per cent., and 20 per cent. CaO and 10, 15, and 20 per cent. $Ca(OH)_2$. The second and third series used only the weaker strengths, 1.8 and 5 per cent., of each. The limes were made up on the basis of actual titrations of the CaO strengths of samples. Table VI gives the result of the tests.

Strengths from 10 to 20 per cent. seem to kill anthrax in from 7 to 10 days, showing that dehairing limes should remove danger of infection. Five per cent. CaO killed in 11 days, 1.8 per cent. suspension from burnt lime killed in 11 days, but when made from hydrated lime it failed to kill in 21 days.

TABLE VI.

Disinfectant action of lime on anthrax skins.

Lime suspension.	Time of exposures in days.													
	At once.	1	2	3	4	5	6	7	8	10	11	12	17	21
Series I:														
Burnt lime CaO 1.8%...	+	+	+	+	+	+	+	+	+	+			+	−
" " " 5%.....	+	+	+	+	+	+	+	+	+	+			−	−
" " " 20%....	+	+	+	+	+	+	+	+	+	−			−	−
Hydrated lime CaO 1.8%	+	+	+	+	+	+	+	+	+	+			+	+
" " " 5% .	+	+	+	+	+	+	+	+	+	+			−	−
" " " 10%	+	+	+	+	+	+	+	−	−	−			−	−
" " " 15%	+	+	+	+	+	+	+	−	−	−			−	−
" " " 20%	+	+	+	+	+	+	+	+	+	−			−	−
Series II:														
Burnt lime CaO 1.8%...	+	+		+		+					−	−		
" " " 5%.....	+	+		+		+					−	−		
Hydrated lime CaO 1.8%	+	+		+		+					+	+		
" " " 5% .	+	+		+		+					−	−		
Series III:														
Burnt lime CaO 1.8%...	+				+		+			+		+	+	
" " " 5%.....	+				+		+			+		0	0	
Hydrated lime CaO 1.8%	+				+		+			+		+	+	
" " " 5% .	+				+		+			+		+	+	

+ = Anthrax Growth. — = No Anthrax.

To demonstrate the difference in quantitative spore reduction in suspensions of the two types, about 20 c.c. of the 1.8 per cent. and 5 per cent. suspensions of burnt lime and of hydrated lime were placed in sterile test tubes with rubber stoppers. To each was added 0.04 c.c. of a heavy anthrax spore suspension and they were vigorously shaken. One loopful from each tube was then plated in agar and after incubation the resultant colonies were counted. These tubes were then kept at room temperature and shaken daily. Table VII gives the results of these tests. Here the spores were not protected by organic matter. This shows a distinct and progressive reduction in the number of viable spores, decidedly more active with the burnt lime samples than with the hydrated lime, but not rapid enough to justify a 12-hour disinfection period for even 5 per cent. CaO. An anthrax colony isolated from the 5 per cent. CaO suspension after ten days proved to be virulent for a guinea pig.

TABLE VII.

Disinfectant action of lime on anthrax spores in suspension.

Series A.

	At once.	3 days.	4 days.	7 days.	9 days.	11 days.
Burnt lime CaO 1.87%........	2,000	1,000	350	34		0
CaO 5%.....................	4,000	480	30	0		0
Hydrated lime CaO 1.875%....	3,500	1,300	750	190		8
CaO 5%.....................	3,000	450	550	170		0

Series B.

	At once.	4 days.	6 days.	10 days.	12 days.	17 days.
Burnt lime CaO 1.875%.......	650	300	150	17	0	
CaO 5%.....................	700	220	115	7	0	
Hydrated lime CaO 1.875%....	500	170	170	16	2	1
CaO 5%.....................	250	160	160	26	0	

The regulations previously referred to require some form of disinfection of uncertified skins unless effluents are disinfected. They recommed either the Schattenfroh salt and hydrochloric acid method, which is quite efficient but which no one in Pennsylvania uses, or the Seymour-Jones mercuric chloride and formic acid method, which also is not used in this State, but they permit the use of lime as above for goat skins and of 1–1,000 mercuric chloride for cattle hides. Tanners in the State claim that they cannot make satisfactory glazed kid from Schattenfroh disinfected skins, but we are informed that all upper leather tanned in Massachusetts is so treated. In actual practice a great variation is found in the carrying out of lime disinfection. Tannery *E* states that they soak all skins received in a 10 per cent. hydrated lime suspension. Tannery *D* soaks all uncertified skins in a 10 per cent. suspension of burnt lime, adding the dry lime after a 12-hour soak in water. Tannery *B* soaks uncertified skins for 36 hours in 3 bushels of lime in 900 gallons of water (about 3.3 per cent.). Tanneries *A, K,* and *L* use hydrated lime, 15 pounds per 100 gallons, the last one soaking all skins received in the lime. Tannery *M* uses 200 pounds of lime in 4,000 gallons (0.6 per cent.). Tannery *H* uses 15 pounds of hydrated lime, 72 per cent. CaO, in 100 gallons, soaking 16 hours. Tannery *N* uses 195 pounds of lime in 600 gallons of water (4 per cent.). Tannery *O* uses hydrated lime, strength not stated. Tannery *J* uses 202–237 pounds of lime per vat, probably under 3 per cent. All of these tanneries are complying with the Bureau regulations, as all disinfection of uncertified skins is supposed to be done under Bureau supervision.

To determine roughly the probable degree of efficiency of lime washing the following laboratory tests were planned. Three one-inch pieces of match stick and three carborundum points about one inch long were boiled in water for 40 minutes to sterilize them and were then covered with a heavy suspension of anthrax spores. They were then dried at room temperature. One of each was immersed in a freshly prepared cream of lime from burnt lime and others treated in a similar manner with a fresh cream from hydrated lime. The six samples were then dried over night at room temperature and afterward dropped into bouillon tubes and incubated for 24 hours. Tubes of melted agar were then inoculated with a loopful from each bouillon culture and plates poured. The two materials were used to represent the two materials, wood and concrete, from which soak vats are made. These tests showed considerable reduction in the number of anthrax colonies developing from the lime-washed samples but not a complete sterilization of the same.

In all of the tests with lime, as will be seen by referring to Tables VI and VII, there was more or less irregularity of action as was to be expected where the disinfecting agent is mostly in suspension rather than in solution.

In addition to the inadequacy and irregularity of lime disinfection shown above, the laboratory tests show that burnt lime is somewhat more active as a disinfectant than is hydrated lime. This is probably due to the fact that the particles of calcium hydrate formed in solution from the freshly slaked lime remain in suspension as a cream of lime while the suspension of preformed hydrated lime settles out very soon and leaves a solution of lime water containing 0.17 per cent. $Ca(OH)_2$ at 10° C. and less at higher temperatures. Any skins not in the sludge at the bottom of the vat are then merely in this weak lime water, except where lime has settled on them. Where lime is used the vats should be plunged frequently so that the skins will be more apt to be kept in contact with the lime in suspension.

Although not among the primary objects of the survey, notes were made in most instances as to the treatment of liquid wastes. The goat skin tanneries now in operation are all in Philadelphia or Chester and all drain into city sewers which empty into the already heavily polluted Delaware or Schuylkill Rivers. As stated above Tannery *G* chlorinates all liquid wastes, but no other goat skin tanneries treat them in any way. The proper disposal of tannery wastes is an unsolved problem. At times they are strongly acid and at times strongly alkaline. So far as anthrax menace goes, the plain soaks are the only dangerous wastes. Chlorine will undoubtedly render them safe but it is rather expensive for the small tannery. The progressive action of strong lime as shown by test would seem to indicate that a prolonged settling period with consequent concentration would care for the danger, provided the acid wastes from tanning liquors did not neutralize the limes. Among the cattle hide tanneries 30 of the 47 employ some form of sedimentation or so-called filtration but usually the plain water, the limes, and the acid vegetable tan liquor containing organic matter are all treated together and it seems probable that the action of the lime is more or less neutralized. If this is so, any anthrax in the waste liquors would probably remain viable in the sludge and serve to infect pasture land when the sludge is used as fertilizer, as is frequently done. This is a problem for the State Health Department and one which I understand they have in contemplation for the future.

As anthrax was isolated from a 24-hour mercuric chloride disinfectant soak, a test was made to show the probable efficiency or lack of efficiency of this method. Small bits of skin were immersed in a 1–1,000 mercuric chloride solution in the usual proportion of skin to solution employed in the tanneries. Tests made at the end of a one-hour soak showed that there was no free mercuric chloride left in solution, as it had all combined with the albuminous material of the skin. Seymour Jones states that when formic acid is combined with the mercuric chloride the latter does not become attached to the skin in this way (1922). His method, so far as we know, has not been used practically in this country. Tilley (1915) and others have shown that it gave practical disinfection only provided neutralization or other treatment is delayed several days. Tilley recommends a 1–1,000 mercuric chloride in 1 per cent. formic acid. The Bureau regulations permit the use of this method provided the hides are held for two weeks after treatment before further use. This would be a great objection to its adoption by American tanneries, though it would make it a practical method of disinfecting hides or skins before shipment to foreign ports. Seymour Jones recommends that its use be adopted as a preliminary for export in the wet salt condition, and that none but wet salted disinfected skins or hides be permitted to enter English ports. If the claims for this method are substantiated and if the tanners are able to make satisfactory glazed kid from skins so treated, it would be an ideal procedure to adopt for this country, solving entirely the question of anthrax hazard from imported skins. The simple wet salting of all imported skins before

TABLE VIII.

Anthrax mortality in tanning industry in Pennsylvania. 1910–1921.

Year.	From cattle hides.		From goat skins.	
	Cases.	Deaths.	Cases.	Deaths.
1910	1	0	2	1
1911	2	2	0	0
1912	2	1	3	0
1913	3	2	3	1
1914	1	0	4	2
1915	3	2	0	0
1916	18	2	4	3
1917	10	3	6	1
1918	15	2	4	0
1919	12	1	3	0
1920	4	1	15	2
1921	2	0	6	0
Total in 12 years	73	16 (21%)	50	10 (20%)
Grand total	123	26 (21%)		

shipment would practically eliminate the anthrax risk for the stevedore and freight handler.

In connection with the survey there were tabulated 123 cases of anthrax in Pennsylvania from 1910 to 1921 traceable to the handling of cattle hides or goat skins. (See Chart IV.) A detailed analysis of these cases will appear in another paper in the Journal of Industrial Hygiene, but Table VIII gives a summary of the cases by years. Among the 73 anthrax cases connected with cattle hide tanneries we have records in 28 of the type of hide being handled at the time of infection. All but 3 of these were cases from handling dry hides; 4 of them were from certified hides; and 2 were from green salted non-packer hides. In 12 cases South American hides were handled, in 2 cases India hides, in 9 cases hides from India or China, in 1 case hides from China and South America, in 1 case hides from Mexico and South America, and in 1 case each hides from Mexico, Cuba, and Texas. It will be recalled that of the four anthrax cultures obtained in these tanneries 3 were from South American and 1 from Indian hides. In goat skin tanneries it is usually not possible to determine definitely the source of skins responsible for infections as most of these tanneries are handling skins from a number of sources at the same time. Four cases, however, were in tanneries handling nothing but wet salted India skins at the time. Of the positive anthrax cultures from these tanneries, 4 were from South American stock, and 5 from Arabian and 1 from Indian stock.

Several tanneries were found to have installed shower baths for the men, but in only one were they so arranged that the men were provided with two dressing rooms, one with lockers for street clothes and one with hooks and provisions for drying wet work clothes. The worker removes his wet clothes, takes his shower and passes out to the locker room to don his street clothes. This greatly lessens the risk of the worker taking anthrax home with him.

The present provisions of the Bureau of Animal Industry for the disinfection of hides and skins are entirely too lax. Mercuric chloride alone should not be permitted. One or two of the tanneries provided immersion bowls of mercuric chloride for the employees to use for hand disinfection. 1–1,000 mercuric chloride will not kill anthrax spores in a 20-minute exposure (Smyth, 1921) and the bowls seen in use were brown with dirt from workers' hands and so had practically no free mercuric chloride in them. The author knows of no efficient hand disinfectant that will kill anthrax spores in the time usually allowed for such hand washing. A thorough vigorous scrubbing with soap and hot water would be of decidedly more benefit than any quick hand immersion that might be used.

Discussion.

The results of the laboratory examination of samples from cattle hide and goat skin tanneries in Pennsylvania, together with a study of the cases of anthrax traceable to them, show that anthrax is a continuing menace to the workers in the receiving, soaking, and liming departments of these tanneries, and that all imported, non-packer hides, and practically all goat skins should be considered as possible sources of infection. Anthrax has been isolated from certified hides and certified hides have been responsible for cases of anthrax infection. Anthrax has been isolated a number of times from certified goat skins and such skins have been responsible for cases of anthrax. Anthrax was found in over half of the goat skin tanneries sampled and cases have been reported in 42 per cent. of the tanneries. In cattle hide tanneries, anthrax has been found by culture in less than 7 per cent., but cases have been reported from almost 40 per cent. When business conditions revive and there is an increased demand for sole leather, no doubt more non-packer hides will be imported and anthrax will again become more prevalent in these tanneries.

Dry hides have been responsible for more cases of anthrax than have green salted hides and the latter have been responsible for some cases. If the importation of dry hides and skins were prohibited, it would undoubtedly lessen the incidence of anthrax though that provision alone would not remove all risk.

If dry skins and hides continue to be imported provision should be made for their disinfection at one or several ports of entry by either the Schattenfroh or the Seymour-Jones method, if satisfactory to the tanners, and for the shipment of such disinfected materials to the tanneries in the wet salt state. Lime disinfectant, if used, should be with at least a 10 per cent. CaO suspension from burnt lime for over 24 hours, and it should be realized that this lessens risk of infection but does not entirely prevent it. Also as a means of lessening infection risk and diminishing the liability of the more or less permanent infestation of soak vats and their surroundings, whitewashing should be liberally and frequently employed for all wood, concrete, or metal work about the store rooms, soak rooms, and beam house. If the Seymour-Jones method of disinfection will give tannable skins, it would be preferable to lime, provided the skins could be held after soaking, and the Schattenfroh salt and hydrochloric acid method would be preferable to either if the tanners could be induced to use it.

Until tanneries cease to receive anthrax-infected materials, they should be required to provide adequate wash fixtures and shower baths for their exposed men with hot and cold water and soap and towels. Towels should be provided by the tanner and they as well as overalls and work shirts should be disinfected with formaldehyde vapor by the tanner before they are allowed to leave the tannery. The worker should not wear his work shoes out of the tannery and it would be best for him to wear shoes or boots of rubber and felt with wooden soles that could be disinfected.

A further study should be made of the proper disposal of tannery wastes. Chlorine gas is too expensive for the small tannery and simple sedimentation will probably not destroy anthrax in the sludge.

No dry sweeping should be permitted in tannery stock and store rooms. The Bureau of Animal Industry regulations for the disinfection of freight cars, stock rooms, etc., and of coverings of bales of skins are excellent, but they should be made to apply for all imported goat skins and all imported non-packer cattle hides.

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DECOMPOSITION OF "FEEDY" SALMON.*

By ALBERT C. HUNTER.

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In a previous study of the bacterial decomposition of salmon, it was found that there is a more or less definite correlation between the total counts of the bacteria present in the flesh and the physical condition of the salmon (3). The salmon used in that investigation were mature salmon of two species caught on their spawning migration. It is well known that salmon on the spawning migration do not consume food of any kind, and it has also been shown that the alimentary tract of such fish is sterile when no food is present (3) (5). This made the problem of bacterial invasion of the dead fish comparatively simple, inasmuch as the infection was solely from the outside inward, and the disturbing factor of partially digested food containing large numbers of living bacteria was absent.

The practice of catching small, immature salmon, not on the spawning migration, the stomach and intestines of which are usually full of food when caught, has recently been established in the salmon industry on the Pacific Coast. As the practice of catching such salmon with hook and line in the open sea off the coasts of Oregon and Washington has grown rapidly in the past few years, it was deemed advisable to extend the study of bacterial decomposition to include a study of such decomposition in the immature, feeding salmon. The objects of the investigations were to determine the rapidity of decomposition in such fish, whether this decomposition is due solely to bacteria, and, if so, what types of bacteria are present This necessitated the determination of the numbers of bacteria present in the muscular tissue and in the stomach and intestinal contents of the salmon in various stages of decomposition, with the view to establishing a correlation, if possible, between the number of bacteria found and the physical condition of the fish. These results, used in comparison with the data previously obtained on the mature salmon, are presented in this paper. Identification of the organisms isolated from the decomposing salmon, now being carried out, will be reported in a later publication.

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* From the Microbiological Laboratory, Bureau of Chemistry, United States Department of Agriculture. Published by permission of the Secretary of Agriculture.

The fish used in these experiments were small, immature salmon of two species, the chinook salmon (Oncorhynchus tschawytsche) and the silver salmon (O. kisutch). They were caught with hook and line or in purse seines off the mouth of the Columbia River and were delivered within 12 hours at Astoria, Oregon, where the experimental work was conducted. The stomachs and intestines of these fish contained food in various stages of digestion. The food for the most part consisted of small herring or small crustacea.

A number of these salmon sufficient for the experiment planned were obtained immediately on delivery at the cannery, and a statement as to the approximate time at which they had been caught was secured from the fishermen. No fish which had been out of water longer than 24 hours were accepted. Since it was planned to examine the fish at 24-hour intervals, it was not necessary to ascertain within several hours the time at which they were caught. It is recognized that the ideal method would have been to accompany the fishermen to the fishing grounds, but under the existing conditions this was not feasible.

At the end of the first 24 hours, one salmon was selected for examination, and the remainder were kept in a large wooden box, with a thermograph, the intention being to reproduce as nearly as possible the conditions under which such salmon would be handled in the industry. The fish were held for five, six and seven days, according to the experiment, or, in other words, until they had reached such an advanced stage of decomposition that they could no longer possibly be considered fit for food. At 24-hour intervals one salmon was taken to the laboratory, its physical condition recorded, and a bacteriological examination made. During our experiments the lowest temperature recorded on the thermograph with the salmon was 49° F. and the highest was 68° F.

In the bacteriological examination total counts of bacteria in the flesh of the back and of the belly and in the stomach and intestinal contents were made. The flesh around the wound or bruise made by the gaff hook or by the single-tine fork with which the fish was handled was also examined bacteriologically to determine whether or not larger numbers and different kinds of bacteria were present in that region because of the breaking of the skin and inoculation from the contaminated instrument.

In obtaining the samples of flesh for bacteriological analysis the same procedure was followed as in the previous investigation (3), except that the present work was done in the laboratory, where a chemical balance was available and the exact weight of the sample was ascertained before diluting it. After obtaining the samples of flesh, the body cavity of the salmon was opened with sterile instruments, the surfaces of the stomach and the large intestine were seared with a red-hot instrument, and small incisions were made through which the samples of stomach and intestinal contents were taken with sterile forceps or spatula. The samples of flesh, stomach, and intestinal contents were thoroughly shaken with sterile sodium chlorid solution and pieces of sterilized broken glass until an emulsion was obtained. The necessary dilutions were plated on glucose agar and incubated for four days at room temperature. Anaerobic cultures were also made from the various samples obtained.

The total counts of bacteria in the various samples are given in Tables I, II, III, IV, and V.

TABLE I.

No. of hrs. out of water.	Bacterial count from back of immature, feeding salmon.			
	Experiment 1.*	Experiment 2.†	Experiment 3.†	Experiment 4.†
	per gram.	*per gram.*	*per gram.*	*per gram.*
24	800	1,030	140	7,200
48	100	600	100	52,000
72	172,500	47,000	3,000	840,000
96	700,000	530,000	56,000	132,000
120	1,000,000	40,000	90,000	10,800,000
144			2,150,000	580,000
168			9,000,000	

* Chinook salmon.
† Silver salmon.

Before discussing the results given in these tables it is desirable to describe the physical condition of the fish from which the results were obtained. In doing so, descriptions of the fish used in experiment 3 may be taken as characteristic of the fish used in the other experiments, thus eliminating a lengthy repetition of descriptive forms.

TABLE II.

No. of hrs. out of water.	Bacterial count from belly of immature, feeding salmon.			
	Experiment 1.*	Experiment 2.†	Experiment 3.†	Experiment 4.
	per gram.	*per gram.*	*per gram.*	*per gram.*
24	19,500	6,000	2,100	3,800
48	270,000	2,200	175,000	1,725,000
72	830,000	1,850,000	1,050,000	400,000
96	700,000	1,380,000	27,000	800,000
120	5,400,000	485,000	299,000	580,000
144			9,100,000	35,000
168			410,000	

* Chinook salmon.
† Silver salmon.

TABLE III.

No. of hrs. out of water.	Bacterial count from flesh around wound in immature, feeding salmon.			
	Experiment 1.*	Experiment 2.‡	Experiment 3.‡	Experiment 4.‡
	per gram.	*per gram.*	*per gram.*	*per gram.*
24	†	122,500	5,300	138,000
48	111,000	8,600	25,250	1,280,000
72	39,750,000	5,500,000	920,000	320,000
96	1,010,000	26,000,000	†	16,000,000
120	5,600,000	†	4,800,000	37,000,000
144			13,850,000	16,000,000
168			500,000,000	

* Chinook salmon.
† Fish entirely without wound or bruise.
‡ Silver salmon.

The external appearance of the salmon out of water 24 hours was considered good, except that the lower jaw was badly torn by the hook and there was one large wound in the back. The eyes were bright and the gills were light pink in color, with the peculiar odor which is characteristic of these fish. The skin was bright and the flesh of the fish was firm. Internally the salmon was also in good condition. All the viscera were firm and there were no offensive odors. There was comparatively little food in the stomach, but the intestines were full of digested food.

TABLE IV.

No. of hrs. out of water.	Bacterial count from stomach contents of immature, feeding salmon.	
	Experiment 3.*	Experiment 4.*
	per gram.	*per gram.*
24......	133,500	470
48......	600	1,780,000
72......	22,000	41,000
96......	3,500	860,000
120......	6,000	1,450,000
144......	39,000	28,000
168......	870,000	

* Silver salmon.

TABLE V.

No. of hrs. out of water.	Bacterial count from intestinal contents of immature, feeding salmon.	
	Experiment 3.*	Experiment 4.*
	per gram.	*per gram.*
24	1,800	4,500
48	80,000	610,000
72	1,750,000	1,700,000
96	1,020,000	8,100,000
120	770,000	2,300,000
144	6,800,000	1,250,000
168	2,950,000	

* Silver salmon.

The external appearance of the 48-hour fish was only fair. The lower jaw was torn, there was a large wound in the back near the head, and a large piece of flesh had been torn out of the back near the tail. The gills were light pink and had a very strong odor which was not particularly offensive. The skin was dry and cracked, and the belly wall was very soft. Internally the fish was not appreciably decomposed. The viscera were firm and not objectionable in odor. There were a few undigested small crustacea in the stomach and a large amount of solid greenish material in the intestines.

The salmon examined at the end of 72 hours showed marked signs of decomposition, both externally and internally. The eyes were red and the gills light pink and very foul in odor. The skin was dry, hard, and cracked, and there was one small wound on the side near the lateral line. The viscera were still firm, although there was a somewhat offensive odor in the body cavity. There was a small amount of slimy liquid in the stomach, and the digested material in the intestines was slimy and light pink in color. The flesh of the salmon was rather soft.

At the end of 96 hours the appearance of the salmon showed it to be in an advanced stage of decomposition. The eyes were very dull and sunken, the gills greenish in color and very foul, the skin was dry and cracked, with the fins dry and stuck to the body. The stomach was exceedingly soft and filled with partly digested herring. All the viscera were very soft, and the pyloric ceca and the intestine were partly disintegrated. The body wall was dark and slimy. The flesh was very soft and pulled away from the ribs.

The salmon examined at the end of 120 hours was decidedly decomposed. Here, again, the eyes were red and dull, gills were grayish-green and very foul, and the skin was dry, hard, and cracked. There was one wound near the tail. The ceca and intestine were disintegrated to such an extent that they were barely recognizable. The stomach contained a large amount of partly digested herring. The body wall was darkened, slimy, and foul.

The 144-hour salmon was even more decomposed than the 120-hour salmon. The eyes and gills appeared much the same, except that flies had deposited eggs in the gills. All the viscera were softened and disintegrated to such an extent that they were barely recognizable. The wall of the stomach was broken through, and the ceca and intestines were almost totally disintegrated. There was an exceedingly foul odor in the body cavity.

The last salmon of this series, held for 168 hours, was almost too far decomposed to be handled. The eyes had disappeared, the gill covers were stuck down, and the gills beneath were white and exceedingly foul. Internally, the only organ recognizable was the stomach. When this fish was opened the disintegrated viscera ran out on the table in the form of a reddish-brown liquid. The flesh was very soft, and the odor surrounding the fish was particularly nauseating.

When the results presented in Tables I to V are considered in connection with the description of the salmon, it is apparent that any attempt to establish a definite correlation between the number of bacteria present and the physical condition of the fish fails. In experiments 1 and 4, reported in Table I, there is an actual decrease in the total count on the last day of the experiment, while in experiments 1 and 3 the numbers of bacteria present certainly are not great enough to account for the condition of the tissue. The results in Table II show a decrease in numbers of bacteria in experiments 2, 3, and 4, and the count of 410,000 bacteria per gram on the seventh day in experiment 3 is insignificant when the exceedingly decomposed condition of the salmon is considered. The results in Table III indicate that the stabbing of the salmon with forks and hooks does introduce large numbers of bacteria into the flesh, but this factor is so variable that the numbers of bacteria in the flesh around the wounds are no indication of the condition of the salmon. Whether or not different kinds of bacteria are introduced by the use of these instruments remains to be determined when the cultures are identified.

The results presented in Tables IV and V, when considered in connection with the physical condition of the viscera of the decomposing salmon, show that no definite correlation exists between the number of bacteria present in the stomach and intestinal contents and the state of decomposition of the viscera. In some salmon in which the viscera were in good condition the total count of bacteria was comparatively high, while in other salmon in which the viscera were badly decomposed the count was small. The condition of the viscera in these cases is apparently so influenced by such factors as the amount and kind of food present, the progress of the digestion, etc., that the determination of the total count of bacteria is very much complicated and may mean little or nothing concerning the decomposition of the salmon. In experiment 4, Table IV, and in experiments 3 and 4, Table V, there is a marked decrease in numbers of bacteria on the last days of the experiments, in spite of the fact that the viscera were so disintegrated as to be unrecognizable.

While there was good growth in all the anaerobic cultures, it was found that no strict anaerobes were present. The organisms isolated from these cultures were found to be facultative.

In order to investigate fully the cause of the decomposition in the immature, feeding salmon, a comparison of the total counts given in the tables is made with total counts on the muscular tissue of the back and belly of the decomposing mature salmon caught on the spawning migration. This comparison is represented graphically in Figs. 1 and 2. The averages of the counts from the back and belly given in experiments 1, 2 and 3, in Tables I and II, were plotted according to the logarithms of the numbers, since the actual numbers are too great to be represented. The averages of the total counts from the back and belly of the mature salmon, as given in the previous paper (3), are also plotted on the same charts. In computing the averages from the mature fish the results from those salmon which were washed on arrival at the cannery have not been included, as they were not handled under conditions analogous to those of the present experiment.

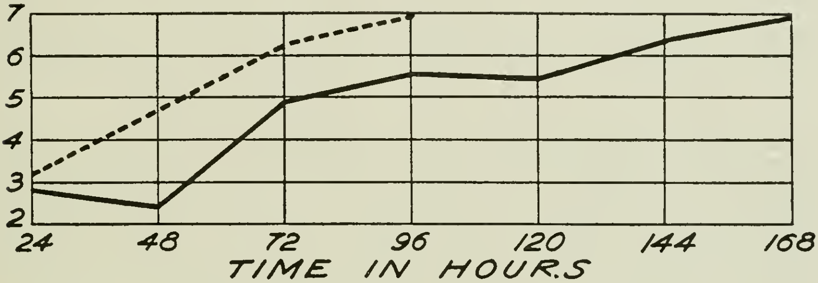

Fig. 1. Chart showing total counts of bacteria from the flesh of the back of mature salmon. Points on the ordinates represent the logarithm of the numbers. The solid line represents counts from the immature, feeding fish; the dotted line, counts from the mature, non-feeding fish.

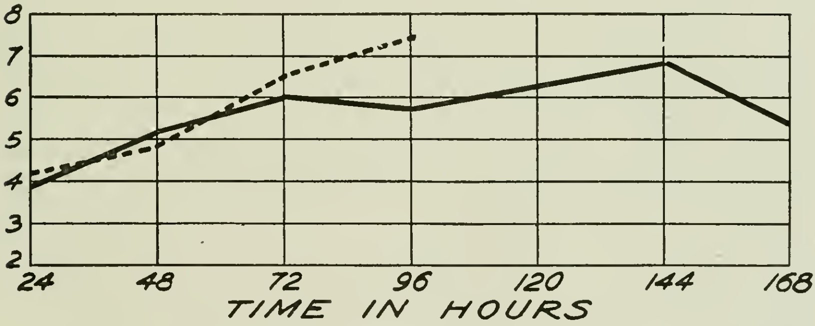

Fig. 2. Chart showing total counts of bacteria from the flesh of the belly of mature and immature salmon. Points on the ordinates represent he logarithms of the numbers. The solid line represents counts from the immature, feeding fish; the dotted line, counts from the mature, non-feeding fish.

Examination of the chart in Fig. 1 shows that the total counts of bacteria from the muscular tissue of the back of the nonfeeding salmon were always greater than those in the feeding fish. The counts in the former show a steady increase from day to day, while the graph made from the counts on the latter is broken by a decrease on the second and fifth days. In the chart in Fig. 2 the graph from the results obtained from the nonfeeding fish fell below the graph representing the feeding fish on the second day, because of an exceptionally low count in the nonfeeding salmon examined after 48 hours. Otherwise, in Fig. 2 as in Fig. 1, the total counts from the muscular tissue of the belly of the nonfeeding fish are greater than those in the feeding fish, and the increase in numbers in the nonfeeding salmon is steady day by day. In the feeding fish there are breaks in the graphs on the fourth and seventh days, due to decreases in the total counts. In appearance, the feeding salmon were much worse than the nonfeeding salmon on the respective days.

Evidently something present in the immature, feeding salmon and not present in the mature, nonfeeding fish causes a softening of the flesh and a disintegration of the viscera, giving all the appearance of a markedly decomposed fish. Müller (4) described experiments which he conducted to show that decomposition in fish can be due to autolysis. He also quoted the unpublished results of Schmidt-Nielsen to show that a fish kept in the refrigerator for 14 days developed an objectionable odor and appearance and that the deep tissue remained sterile. Müller further stated that he was convinced that fish flesh, even without bacterial activity, can undergo, in a purely enzymic manner, such changes that the fish would be considered an undesirable and decomposed food product. It was Müller's conclusion that the "ripening" of meats and the analogous process in fish were caused by enzymes and that while this process in meats improves them for food purposes it changes fish flesh to an undesirable product. Bruns (2) also carried on some investigations with fish in which he partly confirmed the findings of Müller. More recently Browne (1) investigated the decomposition of various kinds of fish stored in ice, and reported that autolysis rather than bacteria appeared to play the most important part.

The results of the work presented in this paper tend to confirm the reports of Müller (4), Bruns (2), and Browne (1) that autolysis may be an important factor in the decomposition of fish. It is not suggested that autolysis alone is responsible for these post-mortem changes in the salmon, but that both bacteria and autolysis play a part. The production of foul odors and such products as indol and hydrogen sulphid are, no doubt, due to bacterial action. The rapid softening of the flesh and the disintegration of the viscera seen in immature, feeding salmon have not been observed in the mature salmon which contained no food, but the tissue of which did contain many more bacteria than were found in the experiments here recorded. Furthermore, it may be that the enzymes exert some antiseptic action on the bacteria, since a decrease in the number of bacteria present during the later days of the experiment was frequently noted, especially in the belly region. While it is possible that this decrease may be due to the lethal action of certain decomposition products, the fact that no such decrease was observed in the mature, nonfeeding salmon leads to the belief that any antiseptic action which takes place is due to the enzymes present.

From these experiments it is concluded that no definite correlation exists between the numbers of bacteria present in the muscular tissue and the physical condition of immature, feeding salmon which are decomposed. Hence, a determination of the total count of bacteria in the flesh of such salmon is not a true indication of its quality. Apparently another agent which assists in the decomposition of the salmon is present. From present knowledge it would seem that this agent is of an enzymic nature. It is evident that a determination of the number of bacteria in the muscular tissue of the back and belly of salmon is of value in judging the quality only in the mature salmon which are caught on the spawning migration and in which the alimentary tract is devoid of food.

The writer wishes to express his appreciation of the advice and helpful suggestions which have been given by Dr. Charles Thom, under whose supervision this work has been done. Throughout the work the writer has been ably assisted by Mr. B. A. Linden, of the Microbiological Laboratory.

SUMMARY.

1. Total counts of bacteria were made in the muscular tissue of the back and belly, around any wound or bruise, and in the stomach and intestinal contents of immature, feeding salmon.

2. An attempt to correlate the total count with the physical condition of the salmon fails.

3. A comparison of the decomposition of mature salmon which are not feeding with the decomposition of the immature, feeding salmon shows that autolysis may take place in the latter.

4. The determination of the total count, in judging the quality of salmon, is of value only when the salmon are not feeding and the alimentary tract is devoid of food.

5. During the first 48 hours out of water under ordinary con-

ditions immature, "feedy" salmon show but little physical evidence of decomposition. From that time on, decomposition is readily detectable by discolored and foul-smelling gills, dull and sunken eyes, dry and cracked skin, softened flesh, and an offensive odor from the whole fish.

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THE LARVAE OF AMERICAN ANOPHELES MOSQUITOES, IN RELATION TO CLASSIFICATION AND IDENTIFICATION.*

By FRANCIS METCALF ROOT.

Plates XIV and XV.

(Received for publication, March 28, 1922.)

Introduction.

The characteristics of *Anopheles* larvae are of interest from two points of view. The field worker, on the one hand, wishes to be able to identify the different Anophelines from larval characters alone, without being forced to resort to the tedious procedure of breeding out adults. He is particularly interested in the distinctions between good malaria carriers and poor ones. The systematic entomologist, on the other hand, desires also to know whether the variations in larval structure may not shed light on the classification of this group of closely related species.

The larval characteristics of the Old World Anophelines are now fairly well known. The publications of Christophers, Stanton, Strickland, Swellengrebel, and Edwards, to mention only a few of the workers in this field, have given to science careful descriptions and figures of the majority of the *Anopheles* larvae of Europe, Africa, and the Orient.

In America, conditions are not quite so satisfactory. The monograph of Howard, Dyar, and Knab describes the larvae of many North and Central American species, and descriptions of *Anopheles* larvae have also been published by Peryassu in Brazil and by Lardé Arthes in Salvador. Unfortunately, none of these authors have based their tabulations of species on the variations of the clypeal hairs, which, according to the Old World authors, furnish the most significant and reliable characters. For this reason, it has been difficult to compare the descriptions of American species with those of Old World forms.

Recently, through the great kindness of Dr. Harrison G. Dyar, I have been permitted to study the collection of larval skins of Anopheline mosquitoes in the United States National Museum, including the specimens on which the larval descriptions in the Monograph were based. In this paper I propose to describe the larval characters which are useful in distinguishing between different species of *Anopheles,* indicate the groups into which the species fall, and give descriptions of these characters for the species which I have been able to examine. For the convenience of field workers, I also include a key or synopsis to the American *Anopheles* larvae, so far as they are known. Besides the material in the National Museum collection, I have also studied considerable numbers of larvae collected by myself in various parts of the eastern United States and in Porto Rico, and have utilized the previous publications of Howard, Dyar, and Knab and of Peryassu wherever it was possible.

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* From the Department of Medical Zoology, School of Hygiene and Public Health, The Johns Hopkins University.

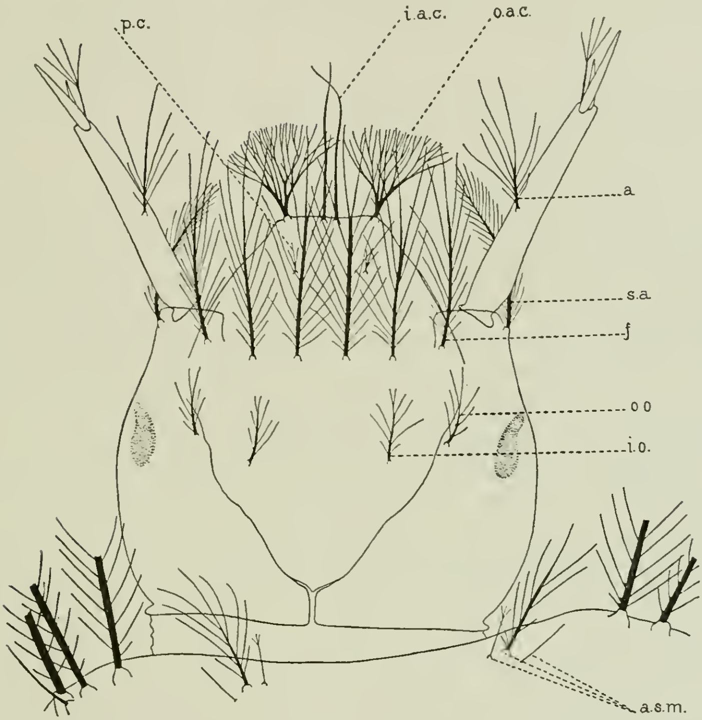
Larval Characters in Anopheles.

The characters which are of use in discriminating between different *Anopheles* larvae are almost entirely the variations in form and position of certain hairs or modifications of hairs. Since the form of these hairs, or rather their degree of development, varies in the different larval stages of the same individual, everything which is said hereafter about the characteristics of the different species must be taken as applying only to the last larval stage, the mature larva. The characters can usually be clearly seen in the living larva. They show up still better in larvae killed and mounted whole in balsam. Many of them can be made out with ease in the cast larval skins, studied in glycerine or balsam mounts. By breeding out adult *Anopheles* in individual vials the larval skin can be preserved for study in connection with the adult which came from it, thus giving an undoubted identification.

The various hairs and similar structures which must be considered in the study of *Anopheles* larvae may be tabulated as follows, using mainly the terminology of Stanton (1915):

- Dorsal Head Hairs.......
    - clypeal hairs.....
        - anterior..
            - inner
            - outer
        - posterior
    - frontal hairs
    - occipital hairs....
        - inner
        - outer
    - antennal hair
    - sub-antennal hair
- Dorsal Thoracic Hairs....
    - anterior sub-median group
    - palmate hairs

Abdominal Hairs........	palmate hairs lateral hairs of segments 4 to 6 lateral comb of segment 8



TEXT-FIGURE 1. Dorsal view of head and anterior edge of thorax of a mature larva of *A. punctipennis*. *i.a.c.*, inner anterior clypeal hair. *o.a.c.*, outer anterior clypeal hair. *p.c.*, posterior clypeal hair. *f.*, frontal hair. *i.o.*, inner occipital hair. *o.o.*, outer occipital hair. *a.*, antennal hair. *s.a.*, sub-antennal hair. *a.s.m.*, anterior sub-median thoracic hairs.

Dorsal Head Hairs.—The location of the head hairs can best be described by reference to text-figure 1, which shows the appearance of the hairs on the dorsal surface of the head and anterior part of the thorax in the common North American species, *A. punctipennis.* For the purposes of our terminology, we imagine the head as divided into three regions—

Clypeus—the narrow portion projecting forward in front of the bases of the antennae.
Frons—the region just between and behind the bases of the antennae.
Occiput—the posterior dorsal region of the head.

1. Inner anterior clypeal hairs. In Fig. 1 (*i.a.c.*) these hairs are long, slender, and simple (*i.e.,* unbranched), arising close together from the anterior edge of the clypeus near the median line and projecting almost straight forward. In other species these hairs may arise much farther apart and may show various other forms. In species which normally have these hairs simple, a forked condition may appear as a rare abnormality. I have seen this in both *A. punctipennis* and *A. quadrimaculatus.*

2. Outer anterior clypeal hairs—a pair of hairs projecting anterolaterally from the edge of the clypeus just above the mouth-brushes. They are often hard to see clearly on this account. In *punctipennis* and many other related species these hairs are thickly dichotomously branched (Fig. 1, *o.a.c.*), forming a flat, fan-shaped tuft. In some species where there are not so many branches, the dichotomous character of the branching can be seen more clearly (*A. strigimacula*). This pair of hairs shows the greatest variety of forms of any of the head hairs and is correspondingly valuable in identification.

3. Posterior clypeal hairs—a pair of hairs on the clypeus but well behind the margin. In *A. punctipennis* (Fig. 1, *p.c.*) they are very short and split into several branches. Their location with respect to the other clypeal hairs may be characteristic even in species where they are otherwise similar (compare *A. pseudopunctipennis* with *A. neivai*).

4. Frontal hairs—these hairs, six in number, are inserted in a more or less irregular line between the bases of the antennae. They extend forward over the clypeal region. In the great majority of *Anopheles* larvae these hairs are long and laterally branched, as they are in *A. punctipennis* (Fig. 1, *f.*). The shortening and reduction of branching which are found in a few species seem to be in some way a response to unusual habitats, for they occur only in species which breed in tree-holes, bamboo joints, or the leaf-bases of epiphytic Bromeliaceae. These species include the members of the Coelodiazesis group (*A. barberi, plumbeus, barianensis* and *culiciformis*), in which the frontal hairs are very short with few or no branches, the *Dendropaedium* group (*A. cruzi, A. neivai*), and *A. asiatica.* In the last three species, the frontal hairs are of normal length but some or all of them lack the usual lateral branches. In *A. fajardoi* the outermost pair of frontal hairs seems to be absent.

5. Occipital hairs—the two pairs of occipital hairs are usually similar and may be considered together. The inner and outer pairs

(Fig. 1, *i.o.* and *o.o.*) are separated by the converging lines which mark the line along which the head-capsule splits at ecdysis. These hairs do not appear to furnish any important characters for the identification of American *Anopheles,* but this may be because they have not been studied extensively.

6. Antennal hair—a hair on the shaft of the antenna (Fig. 1, *a.*) usually located on the inner or dorsal surfaces, from one fourth to one half of the distance from base to apex. Christophers (1913) called attention to the fact that in all the primitive forms (his *Proto-anopheles*) this hair was branched, while in the more specialized forms (his *Deutero-anopheles* and *Neo-anopheles*) it was simple. This relation is certainly the rule, but it is not of universal application. Even in the Orient, the species of the *Coelodiazesis* group have this hair simple, in keeping with the general reduction of the head hairs incident to their tree-hole habitat. The common and widespread South American species usually placed in the ''Cellia'' group have branched antennal hairs. And finally, in *A. pseudo-punctipennis,* I found larval skins from Central America in the National Museum collection which showed a distinctly branched antennal hair, while larval skins from California (originally described as *A. franciscanus*) had the antennal hair simple. In species which have this hair branched, there is considerable variation in the number of branches in different species, as may be seen by comparing the figures of the antennal hairs of *A. albimanus, A. grabhamii,* and *A. strigimacula* (Plate XIV, Fig. 1–3).

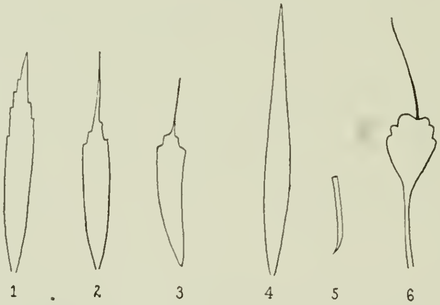
7. Sub-antennal hair—this is the hair which James and Liston call the ''basal hair.'' In most species it is a long feathered hair inserted laterally just behind the base of the antenna and curving forward and downward to lie below the antennal shaft (Fig. 1, *s.a.*). In *A. culiciformis,* of India, this hair has a characteristic clubbed form.

Dorsal Thoracic Hairs.—Of the many hairs on the thorax, only two groups have been carefully studied for purposes of specific discrimination.

1. Anterior sub-median thoracic hairs—two groups of three hairs each, situated near the mid-dorsal line and the anterior edge of the thorax (Fig. 1, *a.s.m.*). The outer hair of each group is always short and simple. In the primitive *Anopheles* the median hair is long and laterally branched, while the inner one is short and only very slightly branched if at all. See figures of these hairs in *A.*

punctipennis (Fig. 1) and in *A. barberi* and *A. grabhamii* (Plate XIV, Figs. 4 and 5). In the more specialized forms, both of the South American and Oriental-African faunas, both the median and inner hairs are long and profusely branched (*A. albimanus,* Plate XIV, Fig. 7). *A. neivai* shows the slightest development of the inner hair which I have found in a member of the specialized South American group (Plate XIV, Fig. 6). *A. fajardoi* shows, as usual, an entirely unique condition, having the inner hair profusely branched on one side only and the median hair very long, forked close to the base, and sparsely branched (Plate XIV, Fig. 8).

2. Palmate hairs—the posterior corners of the thorax bear a palmate hair in many of the Oriental *Anopheles.* Small palmate hairs on the thorax have been reported only in *A. albimanus* and *argyritarsis* among American species. The distribution of the palmate hairs and their use in identification will be discussed in more detail when considering the palmate hairs of the abdominal segments.



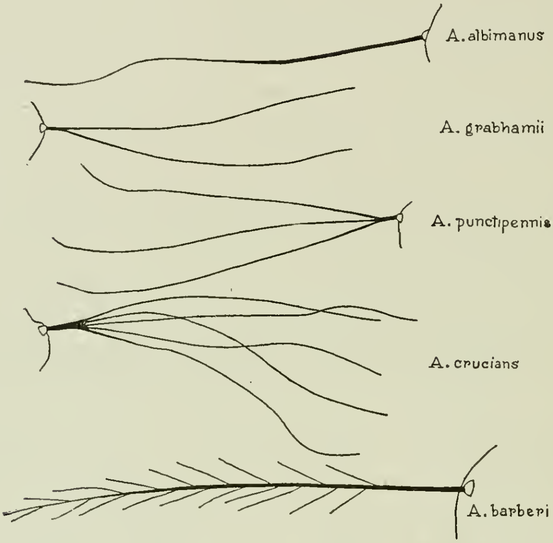
TEXT-FIGURE 2. Individual leaflets of the palmate hairs of the larvae of—

1. *A. grabhamii.*
2. *A. pseudopunctipennis.*
3. *A. schuffneri* (after Swellengrebel).
4. *A. albimanus.*
5. *A. neivai.*
6. *A. fajardoi.*

Abdominal Hairs.—1. Palmate hairs—these peculiarly modified hairs occur in pairs and may be present on the thorax and on abdominal segments 1 to 7. Their location has been extensively used as a diagnostic character, but it is evident that much more variation in this respect occurs than had previously been taken into account. Darling (1910) first pointed out that the larva of *A. albimanus* sometimes possessed palmate hairs on its thorax, in Panama. Recently, Miss Evans has reported the same occurrence in larvae of *A. argyritarsis* from Venezuela. In a large collection of the larvae of *A. albimanus* which I recently made in Porto Rico, this condition is almost universal. Evidently, then, the larval distinction between *argyritarsis* and *albimanus* given in Howard, Dyar, and Knab's monograph will not hold in all cases. Several field men have recently expressed to me their doubt whether the distinction between *A. crucians* and *A. quadrimaculatus*, based on the number of their palmate hairs, will hold good. At any rate, it is clear that the location and number of the palmate hairs must be used with caution as a diagnostic character and only in default of something better. The form of the individual leaflets of these hairs, however, seems to be a character of great value for classification. In the majority of the "Proto-anopheles" the leaflets are lanceolate with a number of irregular notches along the sides near the tip (Fig. 2, 1). In the specialized Old World group, the leaflets have a lanceolate base and a long or short terminal filament, usually separated off by notches (Fig. 2, 3). This condition is not approached by any of the American species, with the exception of *A. pseudopunctipennis* (Fig. 2, 2). In the specialized group, typical of tropical America, the leaflets are slender and smooth, entirely without notches, and either sharply pointed (*A. albimanus*, Fig. 2, 4) or with truncate tip (*A. neivai*, Fig. 2, 5). *A. fajardoi* lives up to its reputation for uniqueness by the leaflet shown in Fig. 2, 6, shaped something like a tennis racket and equipped with a long terminal hair.

2. Lateral hairs of abdominal segments 4 to 6—in all Anopheline larvae, so far as I am aware, the first two abdominal segments bear two very long and strong feathered hairs on each side, while the third segment bears a single hair of the same kind. It has long been known, also, that members of the *Coelodiazesis* and *Dendropaedium* groups bear smaller but still distinctly feathered hairs on segments four to six. Not much attention has been paid to these hairs in other species, perhaps because they are difficult to make out in larval skins which have been mounted in balsam. There are distinct differences in these hairs, in some species at least, which will probably repay study. Figure 3 shows their form in some of the species which I have studied. The examination of a long series of larvae of *albimanus* and *grabhamii* has shown no variations from the condition shown. In *A. punctipennis* and *A. quadrimaculatus* these hairs may be split up into three,

four, or even five branches. The five-branched condition was most common in the comparatively small number of larvae of *A. crucians* which I have examined.



TEXT-FIGURE 3. Lateral hairs from the fourth abdominal segments of the larvae of several species of *Anopheles.*

3. Lateral comb of the eighth segment—the lateral chitinous comb of the eighth segment in *Anopheles* larvae corresponds to the more or less triangular patch of scales found in about the same position in many other mosquito larvae. In nearly all species the comb consists of long and short teeth, irregularly alternated. In species of the *Coelodiazesis* and *Dendropaedium* groups there seems to be a tendency for all the teeth to be long and about of equal length. In *A. neivai* and, according to Christophers, in the Oriental *A. culiciformis* the teeth of the comb have numerous lateral branches.

LARVAL CHARACTERS AND CLASSIFICATION.

The subdivision of the genus *Anopheles* has been carried to an extreme by Theobald, who recognized a large number of genera, distinguished mainly by the form and distribution of scales in the adult. This system took on a new lease of life when Christophers (1913) revised it as the result of splendid detailed studies of adult coloration and larval characters. Even as thus revised, Theobald's classification seems far more detailed than the group warrants and it will undoubtedly be superseded by the simple and natural division suggested by Christophers in 1915 as a result of his studies on the male hypopygium. In this paper I propose to accept Christophers' subdivisions as subgenera, retaining also some of the Theobaldian generic names as convenient handles for referring to particular groups of closely related species. Most of the American species which I have been able to examine fit into this classification very naturally. Some few of the aberrant South American species will require further treatment. *A. fajardoi,* for instance, differs so markedly from all the other species of *Anopheles,* that I am obliged to place it in a special subgenus. A study of the hypopygium of this species may necessitate placing it in a separate genus. The following synopsis shows the grouping indicated for the American species on the basis of larval characters:

Genus Anopheles Meigen.

Sub-genus *Anopheles* Meigen. Type, *A. maculipennis* Meigen.

Leaflets of palmate hairs usually lanceolate, notched near tip.

Antennal hair usually branched.

Inner hair of anterior sub-median thoracic group simple or only very slightly branched.

Coelodiazesis group—characterized by extreme reduction of all head hairs and by the presence of feathered lateral hairs on abdominal segments four to six.

Species known to be included—*A. barberi.*

Anopheles group—characterized by thick dichotomous branching of outer anterior clypeal hairs.

Species known to be included—*A. quadrimaculatus, occidentalis, punctipennis, crucians, grabhamii.*

Species probably included—*A. walkeri, atropos.*

?Patagiamyia? group—characterized by unbranched condition of all clypeal hairs.

Species known to be included—*A. pseudopunctipennis, eiseni.*

Arribalzagia group—characterized by thin dichotomous branching of outer anterior clypeal hairs.

Species known to be included—*A. strigimacula, punctimacula.*

Species probably included—*A. maculipes, apicimacula, mediopunctatus, vestitipennis, pseudomaculipes, intermedius.*

Sub-genus *Nyssorhynchus* Blanchard. Type, *A. argyritarsis* Rob.-Desv.

Leaflets of palmate hairs narrow, without notches, usually ending in a smooth, sharp point.

Antennal hair slightly branched or simple.

Inner hair of anterior sub-median thoracic group profusely branched.

Nyssorhynchus group—characterized by slightly branched antennal hair and branched clypeal hairs. Frontal hairs normal.

Species known to be included—*A. albimanus, tarsimaculata, argyritarsis.*

Species probably included—*A. braziliensis, pictipennis.*

Myzorhynchella group—characterized by simple antennal and clypeal hairs (according to Peryassu). Frontal hairs normal.

Species known to be included—*A. lutzi, parva.*

Species probably included—*A. nigritarsis, gilesi.*

Dendropaedium group—characterized by feathered lateral hairs on abdominal segments four to six and by presence of some unbranched frontal hairs. Antennal and clypeal hairs simple.

Species known to be included—*A. cruzi, neivai.*

Species probably included—*A. bellator, hylephilus,* also possibly *A.* (*Kerteszia*) *boliviensis.*

Sub-genus *Chagasia* Cruz. Type, *A. fajardoi* Lutz.

Leaflets of palmate hairs racket-shaped with a terminal hair.

Antennal hair simple.

Median hair of anterior sub-median thoracic group split into two branches from base.

Only four frontal hairs present.

Species known to be included—*A. fajardoi.*

Larval Characters and Identification.

For the convenience of field workers, I include here a key to the identification of the mature larvæ of American *Anopheles* as far as they are known. As can be readily seen, much remains to be done in completing such a key. The characters at present known only serve to designate whole groups of species in many instances. I have included statements as to the position of the palmate hairs, though in the light of what I have already stated in regard to their use, they should not be relied upon absolutely. The form of these statements can be readily understood, I think. For example, the statement "Palmate hairs on Th, 1–7" means that they are present on the thorax and the first to seventh abdominal segments.

Key to the Known Larvae of American Anopheles Mosquitoes.

1. Outer anterior clypeal hair unbranched .... 2.
   Outer anterior clypeal hair dichotomously branched .... 5.
   Outer anterior clypeal hair split up like a fan at tip—
      A. fajardoi—Palmate hairs on 3–5.
   Outer anterior clypeal hairs with fine lateral branches—
      A. albimanus—Palmate hairs on Th, 1–7 or on 1–7.
      A. tarsimaculatus—Palmate hairs on 1–7.
      A. argyritarsis—Palmate hairs on Th, 1–7 or on 2–7.
2. Lateral hairs of abdominal segments 4 to 6 feathered .... 3.
   Lateral hairs of abdominal segments 4 to 6 not feathered .... 4.
3. Frontal hairs very short and unbranched—
      A. barberi—Palmate hairs on 3–6.
   Frontal hairs of normal length, some of them unbranched—
      A. cruzi—Palmate hairs on 1–7, leaflets sharp pointed.
      A. neivai—Palmate hairs on 2–7, leaflets truncate at tip.
4. Leaflets of palmate hairs slender, smooth, not notched—
      A. lutzi—Palmate hairs on 1–7.
      A. parvus—Palmate hairs on 1–7.
   Leaflets of palmate hairs lanceolate, notched near tip—
      A. eiseni—Palmate hairs on 2–7.
   Leaflets of palmate hairs approaching terminal filament type—
      A. pseudopunctipennis—Palmate hairs on 3–7.
5. Outer anterior clypeal hair with more than twenty ultimate branchlets..... 6.
   Outer anterior clypeal hair with less than ten ultimate branchlets—
      A. punctimacula—Palmate hairs on 3–7.
      A. strigimacula—Palmate hairs on 3–7.
      A. apicimacula—Palmate hairs on 3–7.
6. Inner anterior clypeal hair with fine lateral branches—
      A. grabhamii—Palmate hairs on 3–7.
   Inner anterior clypeal hairs unbranched—

A. punctipennis—Palmate hairs on 2–7.
A. quadrimaculatus—Palmate hairs on 2–7.
A. crucians—Palmate hairs on 3–7, first and last pairs small.
A. occidentalis—Palmate hairs on 3–7, all about same size.

APPENDIX.

Descriptions of Larvae of American Anopheles.

A. barberi Coquillet. Antennal hair simple. Clypeal hairs all short and simple. Frontal hairs all very short and simple or forked. Occipital hairs short and simple.

Inner hair of anterior sub-median thoracic group simple. Palmate hairs on abdominal segments 3 to 6. Leaflets lanceolate, notched toward tip. Lateral hairs of segments 4 to 6 feathered. Comb of eighth segment with long teeth.

A. punctipennis Say. Antennal hair branched. Inner anterior clypeal hairs long, simple, close together. Outer anterior clypeal hairs thickly dichotomously branched. Posterior clypeal hairs very short, three branched. Frontal hairs normal. Occipital hairs branched.

Inner hair of anterior sub-median thoracic group slightly branched at tip. Palmate hairs on abdominal segments 2 to 7, slightly smaller on 2. Leaflets lanceolate, notched toward tip. Lateral hairs of segments 4 to 6 split into 3 to 5 branches. Comb of eighth segment with long and short teeth.

A. quadrimaculatus Say. Larva indistinguishable from *punctipennis.*

A. occidentalis Dyar and Knab. Similar to *A. punctipennis*, but palmate hairs only on segments 3 to 7, all being of equal size.

A. crucians Wied. Similar to *A. punctipennis,* but palmate hairs on segments 3 to 7, those on 3 and 7 slightly smaller. Lateral hairs of segments 4 to 6 split into from 4 to 6 branches.

A. grabhamii Theobald. Similar to *A. punctipennis,* but inner anterior clypeal hairs have fine lateral branches outwardly, palmate hairs occur on segments 3 to 7 only and the lateral hairs of segments 4 to 6 are split into two branches.

A. pseudopunctipennis Theobald. Antennal hair simple or slightly branched. Inner anterior clypeal hairs long, simple, set well apart. Outer anterior clypeal hairs long, simple. Posterior clypeal hairs long, simple, not much farther apart than inner pair of anterior clypeal hairs. Frontal hairs normal. Occipital hairs long and feathered.

Inner hair of anterior sub-median thoracic group slightly branched at tip. Palmate hairs on abdominal segments 3 to 7. Leaflets lanceolate, with a long slender tip. Comb of eighth segment with long and short teeth.

A. *eiseni Coquillet.* Antennal hair branched. Clypeal hairs as in *pseudopunctipennis.* Frontal hairs normal. Occipital hairs simple, forked, or slightly branched.

Palmate hairs on abdominal segments 2 to 7. Leaflets lanceolate, with notches toward the tip. Comb of eighth segment with long and short teeth.

A. *punctimacula Dyar and Knab.* (*A. malefactor* Dyar and Knab.) Antennal hair much branched. Inner anterior clypeal hairs long, close together, with fine side branches outwardly. Outer anterior clypeal hair dichotomously branched, ordinarily with only eight ultimate branchlets. Posterior clypeal hairs short and simple. Frontal hairs normal. Occipital hairs branched.

Palmate hairs on abdominal segments 3 to 7. Leaflets lanceolate, notched toward tip. Comb of eighth segment with long and short teeth.

A. *strigimacula Dyar and Knab.* Indistinguishable from *A. punctimacula.*

A. *neivai Dyar and Knab.* Antennal hair simple. Clypeal hairs all long and simple, inner anterior pair well apart, posterior pair farther apart than outer anterior pair. Frontal hairs of normal length, two inner pairs simple, outer pair feathered toward tip, bare near base. Occipital hairs simple or forked.

Inner hair of anterior sub-median thoracic group long, with numerous lateral branches. Palmate hairs on abdominal segments 2 to 7. Leaflets ligulate with truncate tip. Lateral hairs of segments 4 to 6 feathered. Comb of eighth segment with long teeth feathered on both sides.

A. *cruzi Dyar and Knab.* (*Myzomyia lutzi* Theobald.) According to the description and figure of Peryassu (1921), this species differs from *A. neivai* as follows: All six frontal hairs are shown as long and simple. Palmate hairs on abdominal segments 1 to 7. Leaflets slender, smooth, sharply pointed.

A. *albimanus Wiedemann.* Antennal hair slightly branched. Both pairs of anterior clypeal hairs long, with numerous fine lateral branches. Posterior clypeal hairs fairly long, simple. Frontal hairs normal. Occipital hairs short, slightly branched.

Inner hair of anterior sub-median thoracic group stout and profusely branched. Palmate hairs on thorax and abdominal segments 1 to 7 or on 1 to 7 alone. Leaflets slender, smooth, sharply pointed. Lateral hairs of segments 4 to 6 single. Comb of eighth segment with long and short teeth.

A. *tarsimaculatus Goeldi.* Similar to *A. albimanus,* but palmate hairs have not yet been reported on the thorax.

A. *argyritarsis Robineau-Desvoidy.* Similar to *A. albimanus*, but palmate hairs are reported either on thorax and abdominal segments 1 to 7 or on 2 to 7 only.

A. *lutzi Cruz.* According to the description and figure of Peryassu (1921), the antennal hair is simple. All clypeal hairs simple. Frontal hairs normal. Occipital hairs branched.

Palmate hairs on abdominal segments 1 to 7. Leaflets slender, smooth, sharply pointed.

A. *parvus Chagas.* Indistinguishable from *A. lutzi,* as far as I can tell from the data given by Peryassu.

A. *fajardoi Lutz.* Antennal hair simple. Clypeal hairs very long and stout, split up like the sticks of a fan near tip. Frontal hairs four in number, all long and feathered. Occipital hairs branched.

Inner hair of anterior sub-median thoracic group long, with many branches on one side only. Middle hair of same group split into two long branches, each with sparse lateral branches. Palmate hairs on abdominal segments 3 to 5 only. Leaflets racket-shaped, notched outwardly, with a long terminal hair.

EXPLANATION OF PLATES.

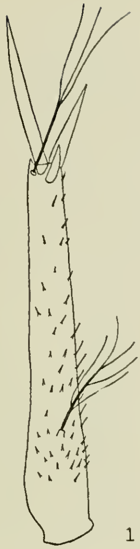
PLATE XIV.

1. Antenna of larva of *A. grabhamii.*
2. Antennal hair of larva of *A. strigimacula.*
3. Antenna of larva of *A. albimanus.*

4 to 8. Anterior sub-median thoracic hairs of larvae of—

4. *A. barberi* (right side).
5. *A. grabhamii* (right side).
6. *A. neivai* (left side).
7. *A. albimanus* (right side).
8. *A. fajardoi* (right side).

PLATE XIV.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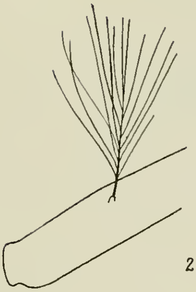
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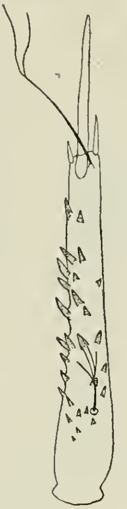
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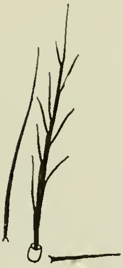
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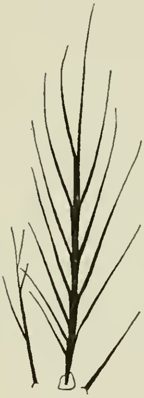
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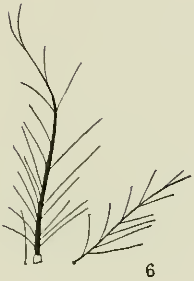
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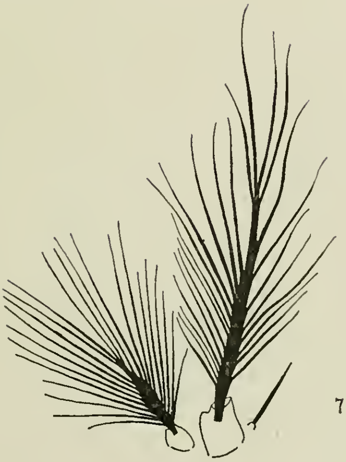
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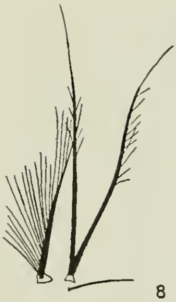
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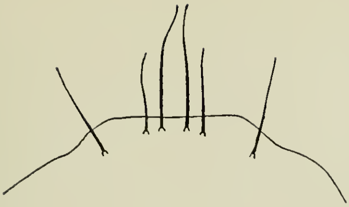
PLATE XV.



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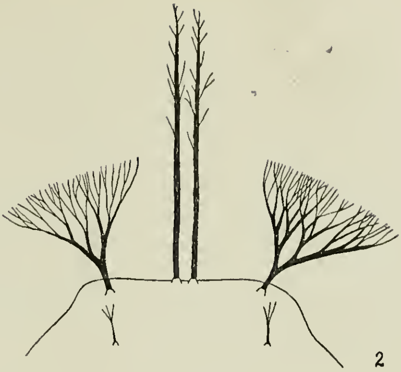

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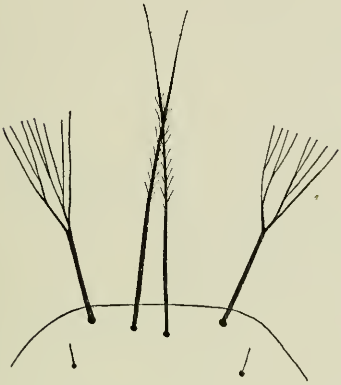

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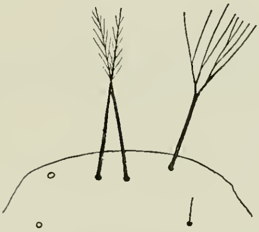

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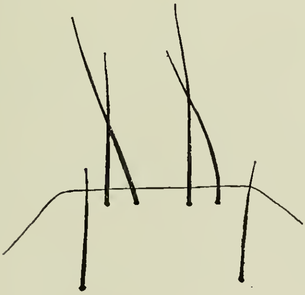

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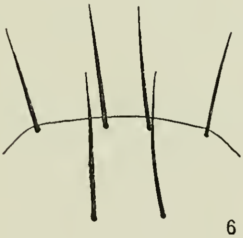

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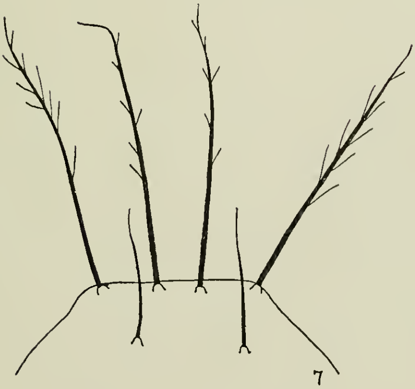

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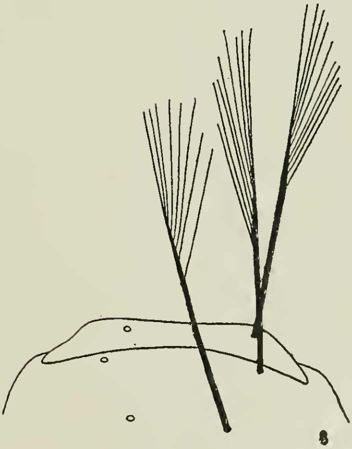

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PLATE XV. Clypeal hairs of the larvae of—

1. *A. barberi.*
2. *A. grabhamii.*
3. *A. strigimacula.*
4. *A. punctimacula.*
5. *A. neivai.*
6. *A. pseudopunctipennis.*
7. *A. albimanus.*
8. *A. fajardoi.*

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NOTES ON MOSQUITOES AND OTHER BLOOD-SUCKING FLIES FROM PORTO RICO.*

By FRANCIS METCALF ROOT.

(Received for publication, February 27, 1922.)

INTRODUCTION.

As the most accessible tropical dependency of the United States, the island of Porto Rico is of the greatest interest to those in our country who are working in Tropical Medicine or in Medical Zoology. During the summer of 1921 an expedition from the School of Hygiene and Public Health went to Porto Rico with the idea of learning just what opportunities the Island offered for study and research in Medical Zoology. Beside making this survey, the leader of the expedition, Dr. R. W. Hegner, head of the Department of Medical Zoology in the School, made studies of the parasitic Protozoa of man and lower animals, and the author of this paper devoted much of his time to collecting and rearing mosquitoes and other blood-sucking flies. Since these observations show that the mosquito fauna of the island is very imperfectly known, the following notes have been published, and in order to make them more useful to anyone who may be interested in the island fauna, an attempt has been made to collect and summarize the previous records for the Island.

I wish to acknowledge my great obligations to Dr. A. Ruiz Soler, at that time Commissioner of Health of Porto Rico, and Dr. R. B. Hill and Mr. H. W. Green of the International Health Board for their interest in and assistance to my work while in the Island, and to Dr. Harrison G. Dyar, Dr. J. R. Malloch, and Dr. James S. Hine for assistance in identifying specimens after my return to the States.

PREVIOUS RECORDS.

The following list includes all the records of blood-sucking flies from Porto Rico which I have been able to find. The initials in parentheses following the names of the various species refer to the authors who have published the records. The various publications with the appropriate initial prefixed are listed below.

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* From the Department of Medical Zoology, The School of Hygiene and Public Health, The Johns Hopkins University.

(C) Coquillet, D. W.
    1900. Report on a collection of Dipterous Insects from Puerto Rico. Proc. U. S. National Museum, XXII, 249–270.
(H) Howard, Dyar, and Knab.
    1912. The Mosquitoes of North and Central America and the West Indies. Pub. No. 159, Carnegie Institution of Washington.
(M) Malloch, J. R.
    1914. American Black Flies or Buffalo Gnats. U. S. Bureau of Entomology. Technical Series No. 26.
(R) Roeder, Victor von.
    1885. Dipteren von der Insel Porto Rico, etc. Stettiner Entomol. Zeitsch. 337–349.
(T) Tower, W. V.
    1921. A Mosquito Survey of Mayaguez. Porto Rico Agr. Exp. Sta. Circular No. 20.

List of Blood-sucking Flies Previously Reported from Porto Rico.

Family Chironomidae—	"*Ceratopogon*" *punctipennis* Williston (C).
	"*Ceratopogon*" *sequax* Williston (C).
Family Simuliidae—	*Simulium quadrivittatum* Loew (M).
Family Culicidae—	*Anopheles albimanus* Wiedemann (R) (H) (T).
	Anopheles grabhamii Theobald (H) (T).
	Megarhinus portoricensis Roeder (R) larvae in tree-holes.
	Aedes portoricensis Ludlow (H).
	Aedes mediovittata Coquillet (H) larvae in tree-holes.
	Aedes aegypti Linn. (H) (T).
	Psorophora jamaicensis Theobald (H).
	Culex quinquefasciatus Say (H) (T).
	Culex toweri Dyar and Knab (H).
	Culex bisulcatus Coquillet (H).
Family Tabanidae—	*Chrysops costatus* Fabr. (R).
Family Muscidae—	*Stomoxys calcitrans* Linn. (R) (C).
Family Hippoboscidae—	*Ornithoctona erythrocephala* Leach (C) on sparrow hawk.
Family Streblidae—	*Trichobius dugesii* Townsend (C) on bats.
	Aspidoptera busckii Coquillet (C) on bats.
Family Nycteribiidae—	*Pterellipsis araneae* Coquillet (C) on bats.

SPECIES OBTAINED BY THE AUTHOR.

All my own collecting was carried on in the narrow coastal plain which surrounds the Island, lack of time preventing any attempt to become acquainted with the fauna of the mountainous interior. The principal collecting points were Rio Piedras and Martin Pena, near the capital, San Juan, on the rainy north coast, and the Central Aguirre, a large sugar plantation between Guayama and Salinas, on the dry south coast.

Family Chironomidae—Culicoides furens Poey.—(Syn. *C. maculithorax Williston.*)—The common "sand-fly" of the coastal region. A small species with maculated wings and a yellow-brown thorax with many small darker spots. Bites man very readily in the evening and even during the middle of the day in shade. The bite is very painful and sometimes produced small swellings lasting for several days and itching slightly. These swellings were not discolored, however. Collected while biting man at Rio Piedras (July 12, August 14, 24, 26), Martin Pena (July 14, August 4, 16, Sept. 1), Aguirre (August 18, 20).

Family Simuliidae—Simulium quadrivittatum Loew.—Not as numerous in the coastal plain as *Culicoides furens.* Bites man readily even in hot sunlight in the middle of the day. The bite was absolutely painless to me and caused no swelling or itching later. Several drops of blood flowed from the wound and when the clot which they formed was removed it disclosed a bright red spot marking the seat of the bite. In about half an hour this spot became surrounded by a pink area about one centimeter in diameter which faded out gradually, persisting several days. Collected while biting man at Rio Piedras (July 13, August 12, 31, September 4).

Family Culicidae—Anopheles albimanus Wiedemann.—Probably common throughout the coastal plain. Adults attack man readily in the evening. Larvae were found in many different kinds of pools, swamps, irrigation ditches, etc., often unshaded but usually with some aquatic vegetation. More abundant near the coast and lagoons than a few miles inland. Adults or larvae collected at Rio Piedras (July 7, 18, August 12), Martin Pena (July 14, 15, August 4, 9, 23, 25, Sept. 1, 3), Aguirre (June 30, August 18, 19, 20).

Anopheles grabhamii Theobald.—Probably found throughout the coastal plain, but in smaller numbers than *A. albimanus.* Attacks man readily in the evening. Breeding places much more local and difficult to find than those of *albimanus.* The places where I found larvae were all well shaded, with considerable aquatic vegetation. Larvae collected at Martin Pena (August 4, 9, 25, Sept. 3), Aguirre (August 18).

Uranotaenia socialis Theobald.—Larvae of this species were always present in a small swampy ditch in a young cane-field near Rio Piedras. This ditch contained a good deal of Spirogyra. Larvae collected at Rio Piedras (June 25, July 6, August 12, Sept. 5).

Uranotaonia lowii Theobald.—Larvae were found in grassy meadow pools kept filled for some time by the frequent rains. Adult collected in house, Rio Piedras (August 26). Larvae collected at Rio Piedras (July 13, 19).

Aedes (*Stegomyia*) *aegypti Linn.* (Syn. *Aedes argenteus Poiret; Aedes calopus Meigen; Stegomyia fasciata Theobald.*)—Very common in and around houses everywhere in the coastal plain, biting by day and in the evening. Larvae swarming in artificial containers about houses. Adults or larvae collected at Rio Piedras (July 2, 10, 12, August 3, 6, 10), Aguirre (June 30, August 18, 19), Quebradillas (June 29), Ponce (June 29, 30).

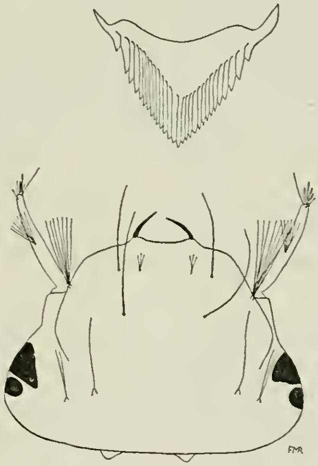

FIG. 1. Head of larva of *Aedes condolescens?* Dyar and Knab. Also outline of mental plate, much enlarged.

Aedes (*Taeniorhynchus*) *portoricensis Ludlow.*—Probably only a geographic race of *Ae. taeniorhynchus Wied.* Larvae were found in large numbers in temporary rain pools near a large lagoon and adults were taken while biting man by day in a mangrove swamp. Adults or larvae collected at Rio Piedras (July 7, 18), Aguirre (August 18).

Aedes (Ochlerotatus) ?condolescens Dyar and Knab?—*Aedes* larvae which may be those of this species were found in temporary rain pools near Rio Piedras on August 26, in company with larvae of *Psorophora jamaicensis*. Since they all died before pupating, it is impossible to be sure of the identification. A description of the mature larva follows:

Larva, Stage IV.

- Head—large, rounded, wider than long, widest just behind eyes.
    - Antennae small, slender, uniform, slightly curved, sparsely spined; a short tuft of about six hairs at middle; tip with a long hair and four short, stout spines.
    - Dorsal head hairs in three pairs. The two outer pairs long and unbranched, the inner pair very short, three-branched. Ante-antennal hair a long tuft of eight or nine hairs.
    - Mental plate triangular, ridged outwardly, with a small central tooth and fourteen teeth on each side, the basal ones becoming broader.
- Body—with the skin minutely spiculate.
- Thorax—wider than long, with many long hair tufts.
- Abdomen—rather short and stout, not tapering decidedly.
    - Lateral hairs, three on first segment, two on second, one on third to sixth.
    - Sub-dorsal hairs, two on first to third and on seventh segment, one on fourth to sixth.
    - Tracheal tubes very broad, slightly swollen in each abdominal segment and much expanded in thorax.
- Air tube—stout, tapered slightly in outer half, about two and one half times as long as wide.
    - Pecten running to middle of air tube, composed of ten to seventeen teeth, the first two usually very small. Single pecten tooth a long spine with one large branch at basal third and sometimes two smaller ones basal to the first.
    - A single pair of hair tufts just beyond middle of air tube, each composed of about eight hairs.
- Lateral comb of eighth segment—a broadly triangular patch of about twenty-four scales. Single scale short, rounded, thinly chitinized, evenly fringed with spines posteriorly.
- Anal segment—about one and one half times as long as wide, broadly ringed by the plate. Dorsal hairs consist of one long, strong hair and a shorter brush on each side. Lateral hairs single. Ventral brush short but well developed, confined to the barred area.
- Anal gills—long, over one and one half times as long as the segment, tapered to a point.

The larva runs out to *Aedes euplocampus* (a mainland species, Mexico to Panama) in Howard, Dyar, and Knab's table. It is evidently closely allied both to this species and to *Aedes scapularis*, but differs from *scapularis* by not having the skin "coarsely pilose." The only differences from *euplocampus* which I can find by comparison with Howard, Dyar, and Knab's figure and description are that in that species the pecten teeth are said to be "a long spine with four or five irregular basal branches," and the inner pair of dorsal head hairs are shown as only two-branched.

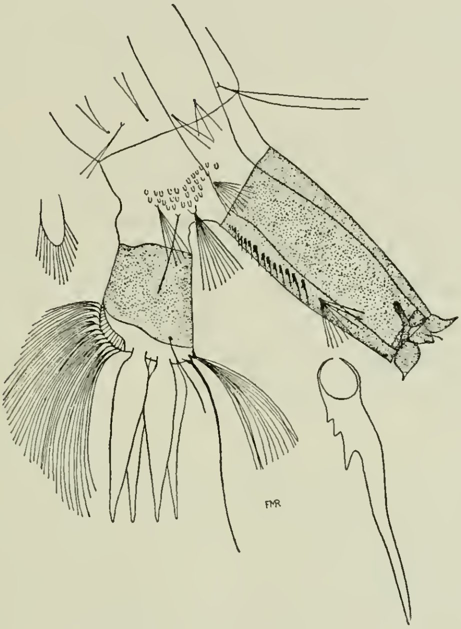

FIG. 2. Seventh and eighth abdominal segments of larva of *Aedes condolescens?* Dyar and Knab. Also outlines of single pecten tooth and single comb scale, much enlarged.

Psorophora jamaicensis Theobald.—Larvae of this species were found in temporary rain pools at Rio Piedras (August 26, 31) and in a recently flooded irrigation ditch at Aguirre (August 18).

Culex (*Culex*) *quinquefasciatus Say.* (Syn. *Culex fatigans Wied.*) —Common everywhere in the coastal plain, breeding in all sorts of artificial containers near houses and biting man readily in the evening. Adults or larvae collected at Rio Piedras (June 26, July 9, August 3, 9, 10, 30), Aguirre (June 30, August 18, 20), Quebradillas (June 29), Ponce (June 29, 30).

Culex (*Culex*) *nigripalpus Theobald, variety similis Theobald.*

(Syn. *Culex microsquamosus Grabham; Culex regulator Dyar and Knab; Culex carmodyae Dyar and Knab; Culex prasinopleurus Martini.*)—Larvae of this species were found in small numbers in temporary meadow pools at Rio Piedras (July 13) and in enormous numbers in a ditch highly polluted with sewage at Fajardo (August 29).

Culex (*Melanoconion*) *atratus Theobald.* (Syn. *Culex falsificator Dyar and Knab.*)—A single male of this species was bred from a pupa collected in a semi-permanent roadside swamp at Martin Pena (July 14). Other larvae and pupae collected at the same time and place all produced *C. borinqueni.*

Culex (*Choeroporpa*) *borinqueni*, new species. (Borinquen is the old Indian name for Porto Rico.)—The commonest "wild" *Culex* of the Porto Rican coastal plain has been pronounced a new species by Dr. Dyar. This species was found breeding in all sorts of ditches, slow streams, pools, and marshy places, often in company with *Anopheles albimanus.* Larvae were collected at Rio Piedras (July 7, 13, 18), Martin Pena (July 14, August 4, 9, 12, 23), Aguirre (August 19). A description of the species follows:

Female.

Head—Proboscis moderate, slightly enlarged at tip; labellae conically tapered; vestiture black with bronzy reflections; setae small, curved, black, more prominent on labellae.

Palpi short, about one sixth as long as proboscis; vestiture black with bronzy reflections.

Antennae moderate, the joints sub-equal, rugose, coarsely pilose, black. Hairs of whorls sparse, moderate, black. Tori sub-spherical with a cup-shaped excavation, dark brown.

Clypeus rounded triangular, bare, black.

Eyes black.

Occiput clothed on sides and along eye-margins with broadly ovate, whitish scales. Scales of center and posterior region narrow, curved, whitish. Among the whitish scales project numerous upright, forked, black scales. A row of setae along margin of eyes.

Thorax—Prothoracic lobes elliptical, remote dorsally, with numerous setae.

Mesonotum blackish, vestiture of narrow, curved, bronzy scales; bristles stiff, coarse, brownish.

Scutellum trilobate, blackish, clothed with pale brown scales; each lobe with a tuft of coarse, dark-brown bristles.

Postnotum elliptical, blackish, bare.

Pleurae light brown with patches of broad, whitish scales and brown bristles.

Abdomen—sub-cylindrical, depressed, truncate at tip.

Dorsal vestiture of broad black scales with bronzy reflections; tips of segments with coarse, brown hairs. A row of lateral, basal, segmental, whitish spots.

Ventral vestiture of dark scales with basal, segmental, whitish bands.

Wings hyaline—Petiole of second marginal cell about one fourth as long as cell.

Petiole of second posterior cell about one half as long as cell. Basal cross-vein about three times its length from anterior cross-vein.

Wing scales of two types, (1) scales broadly to narrowly obovate, truncate at tip, closely appressed to the vein; (2) scales narrowly obovate to ligulate, outstanding. All wing scales black with bronzy and bluish reflections. Scaling of type (1) is found on costa, subcosta, dorsal side of first vein and third vein, ventral side of stem of fourth vein and dorsal side of outer half of anterior branch and all of posterior branch of fourth vein, dorsal side of fifth vein.

Scaling of type (2) is found on ventral side of first vein, both sides of second vein, ventral side of third vein, dorsal side of stem and inner half of anterior branch of fourth vein and ventral side of outer half of anterior branch and all of posterior branch of fourth vein, ventral side of both branches of fifth vein, and both sides of sixth vein.

Halteres—pale.

Legs—moderate; femora brilliant white below except at tip; knees brownish; legs otherwise covered with appressed, black scales with bronzy and bluish reflections.

Claw formula—0.0–0.0–0.0.

Length—Body about 2.5 mm. Wing about 3 mm.

Male.

Head—Proboscis straight, slender, slightly enlarged at tip, black scaled with bronzy reflections.

Palpi exceeding proboscis by a little more than the length of the last joint. Apical portion with long hairs; vestiture of black scales with bronzy reflections.

Antennae long-plumose.

Coloration as in the female.

Wing scales in general distributed as in the female. The scales tend to be shorter and broader, most of the appressed scales being broadly obovate, and the outstanding scales narrowly obovate. Almost no ligulate scales.

Abdomen sub-cylindrical, somewhat enlarged apically, with numerous long bristles.

Male genitalia—Clasp filament narrowly snout-shaped, the spine widened and appendiculate. Pile short and scanty, situated near tip.

Outer division of lobe of side-piece slenderly columnar, widened at tip; a short inner arm bearing a long, stout filament with recurved tip and a short, curved, pointed filament, arising from the base of the arm and bearing a membranous web on one side.

Middle filament long, with leaf-like expansions on both sides at apex, the expansion on one side extending toward the base beyond the middle of filament.

Tip of outer division of lobe of side-piece with a large, broadly expanded leaf and four shorter filaments, flattened and clubbed at tip, which arise serially along the stem of the outer division of the lobe.

Inner division of lobe of side-piece with the arms well separated, outer arm longer than inner, each bearing a long, stout filament with expanded, hooked tip.

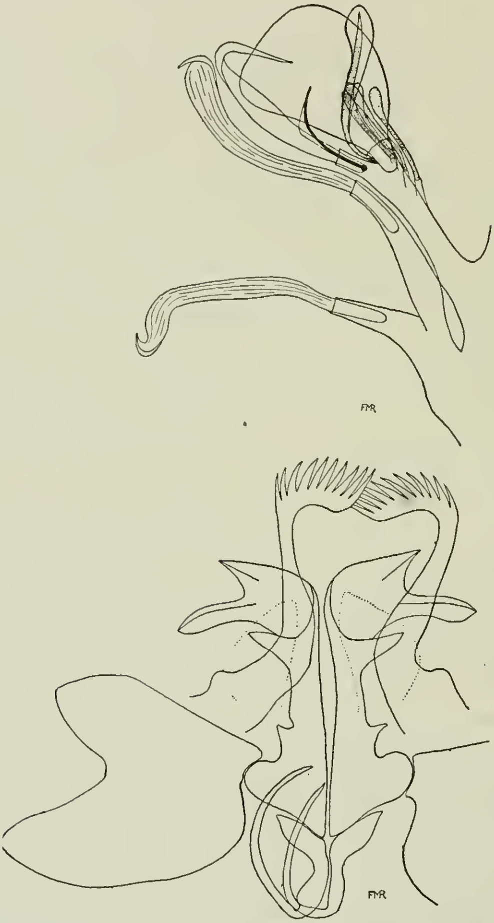

Fig. 3. Male genitalia of *Culex* (*Choeroporpa*) *borinqueni* n. sp. Lobe of side-piece above and basal structures below.

Second uncal plate bifid, both limbs pointed; a stout, almost straight, horn projecting outward from stem near tip.

Articulated plate large, oval, deeply emarginate outwardly.

Basal hooks long, slender, curved, with pointed tips.

Basal appendages large, elliptical, oblique, with many rather long setae.

Harpes comb-like, with ten or eleven stout, strong teeth.

Larva, Stage IV.

Head—large, rounded, wider than long, widest through eyes.

Antennae long; a tuft of over twenty sparsely feathered hairs at outer third, the part beyond slender. Shaft spinose. A short spine and a long stout hair at tip, two more long, stout hairs arising in a notch just below tip.

Dorsal head hairs in three pairs. Two anterior pairs single, outer very long and strong, inner short and delicate. Posterior pair forming short tufts of about five hairs each.

Ante-antennal hairs forming long tufts of about twelve sparsely feathered hairs.

Mental plate triangular, with a large, dome-shaped, central tooth and four teeth on each side, the basal one very small.

Body—with the skin densely pilose.

Thorax—rounded, wider than long, with many tufts of long, stout, sparsely feathered hairs.

Abdomen—long, slender, tapering.

Lateral hairs in threes on segments one, two, and four to six, in fours on segment three. Sub-dorsal hairs in fives or sixes.

Tracheal tubes slender, nowhere expanded.

Air tube about five and one half times as long as wide; gradually and slightly tapered outwardly.

Pecten of about fifteen long, slender teeth, smaller at base; confined to basal third of air tube. A single pecten tooth is a long, slender spine, fringed on inner side with many delicate teeth.

Ventrally five pairs of hair tufts beyond pecten, none out of line,

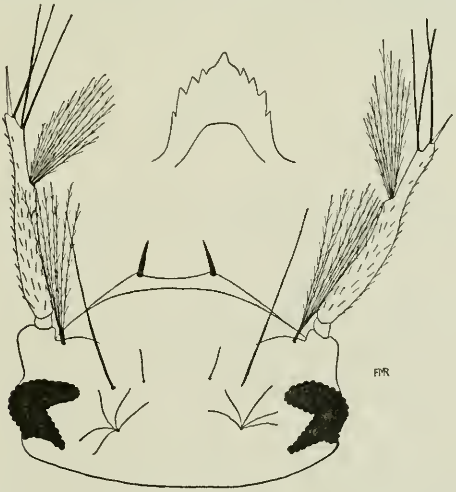

FIG. 4. Head of mature larva of *Culex borinqueni* n. sp. Also outline of mental plate, much enlarged.

each composed of six or seven long, sparsely feathered hairs. Basal two tufts longest, outer three progressively shorter.

Dorsally a small tuft of two hairs between first and second ventral tufts and a small tuft of three hairs between fourth and fifth ventral tufts.

Lateral comb of eighth segment composed of twenty-four scales in two irregular rows, forming a broadly triangular patch. Single scale a long slender spine fringed on both sides with many delicate teeth.

Anal segment about twice as long as wide, ringed by the plate. Dorsal hairs, two long, stout hairs and a shorter hair on each side. Lateral hair single, very fine. Ventral brush long and well developed, confined to the barred area. Anal gills much shorter than the segment, tips rounded.

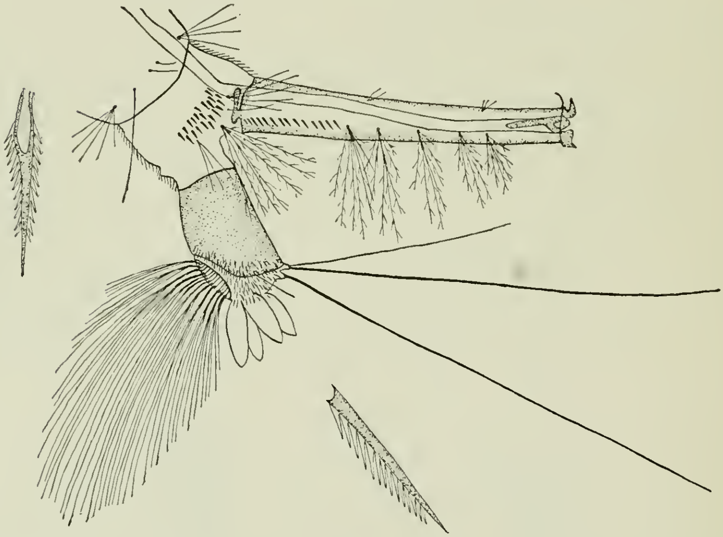

Fig. 5. Seventh and eighth abdominal segments of mature larva of *Culex borinqueni* n. sp. Also single pecten tooth and single comb scale, much enlarged.

This species comes nearest to *Culex* (*Choeroporpa*) *leprincei* (syn. *C. trachycampa*) from Panama, and runs out to that species in Dr. Dyar's genitalic key (Ins. Insc. Men., 1920, Vol. VIII, page 79). The adult is indistinguishable except by the male genitalia. These differ especially in the shape of the third tooth of the second uncal plate, which is narrower, more curved, and horn-like in *leprincei,* and in the position of the filaments near the leaf on the tip of the outer division of lobe of side-piece, which are clumped together in *leprincei,* not inserted serially along the stem. The larva agrees exactly with the description of that of *leprincei*. Possibly the mental plate (which is not described or figured for *leprincei* in Howard, Dyar and Knab's Monograph) may prove to be characteristic.

Culex sp.?—Culex larvae of three other species were taken during my collections without being bred out to adults. One of these is very probably the larva of *Culex atratus*. The other two, one found in an open crab-hole, the other found in small numbers in the same roadside swamp which yielded large numbers of *Anopheles albimanus* and *Culex borinqueni,* are unidentifiable and will not be described.

Deinocerites cancer Theobald.—Probably found throughout the coastal plain near the ocean and the lagoons, where crab-holes occur. Adults or larvae were collected at Rio Piedras (July 7, 18), Martin Pena (August 16, 25), Aguirre (August 20).

Family Tabanidae—Chrysops costatus Fabricius.—Collected only in and around the edges of mangrove swamps. Called ''mosca de manglar'' (mangrove fly) by the Porto Ricans. Attacks man readily by day, inflicting a painful bite. At Aguirre on August 20, a big green-and-black dragonfly (probably *Lepthemis vesiculosa* Fabr.) dashed at my head and captured a specimen of this species which was buzzing about it. Collected at Martin Pena (August 25) and Aguirre (August 20).

Family Muscidae—Stomoxys calcitrans Linn.—Common everywhere about Rio Piedras, feeding on cattle, horses and goats. Not seen to attack man. Adults collected at Rio Piedras (August 3, 6, 12, 23).

A CRITIQUE OF THE SUPPOSED RODENT ORIGIN OF HUMAN GIARDIASIS.*

By CHARLES E. SIMON, B.A., M.D.

(Received for publication, April 20, 1922.)

Plate XVI.

Introduction.

In his publication of 1881, Grassi expressed the opinion that human giardiasis might possibly be of rodent origin. He was led to this conclusion by the discovery that giardias occur quite commonly in various representatives of the sub-family Murinae (*Mus musculus, Mus decumanus,* and *Mus sylvaticus*), as well as in the common meadow mouse, *Arvicola arvalis,* of the district of Italy (Lombardy) where he carried on his investigations, and that, owing to the habit of the peasants of keeping their bread in open garrets its contamination with the dung of some of these rodents was of common occurrence. Grassi's suggestion was, however, only a suggestion, and unsupported by any actual facts. It has nevertheless been handed down in the literature practically to the present day.

Inasmuch as human giardiasis is at times associated with chronic diarrhoea and is in the opinion of some observers its actual cause, it seemed of importance to ascertain the manner in which the infection is spread, and hence to investigate amongst the various possibilities also the validity of Grassi's hypothesis.

To this end, it became necessary to determine (1) whether the giardia which is found in man is morphologically identical with the form or forms which occur in those rodents which might possibly enter into consideration, viz., the common house mouse, the Norway rat and certain types of meadow mice, viz., *Arvicola* in Europe and *Microtus* in North America; (2) to ascertain whether the various types of giardia are non-specific for their hosts, e.g., whether the human form can live in the intestine of the rat, or the rat form in the intestine of the mouse; (3) if host specificity does not exist, whether a given type after transference from its usual host to a host of a different species retains its original morphological characteristics.

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* This is the second of a series of papers on the genus *Giardia* from the Department of Medical Zoology of the School of Hygiene and Public Health of the Johns Hopkins University. The first paper of the series was published in the American Journal of Hygiene, 1921, vol. I, pp. 440–482.

Material and methods

The writer's work is based upon the study of the following forms:

(*a*) Trophozoites and cysts of the type of *Giardia* which is found in man, obtained from five different individuals. The results of the morphological and statistical study of this material have already been published under the title "*Giardia enterica:* A parasitic intestinal flagellate of man" (Simon 1921).

(*b*) Trophozoites and cysts of the type of *Giardia* which Kofoid and Christiansen found in *Microtus californicus californicus* (Peale), and which these writers have named *Giardia microti* (1915). I owe specimens from three different animals of this species to the courtesy of Dr. W. C. Boeck.

(*c*) Trophozoites and cysts of the type of *Giardia* which I found in *Microtus pennsylvanicus acadicus.* These animals were obtained at Chester, Nova Scotia. The organism in question is apparently identical with the *Giardia microti* of California. The statistical results of the study of this type are based upon specimens obtained from five different hosts, while the general morphological study of the organism has reference to specimens derived from 26 different hosts.

(*d*) Trophozoites and cysts of the type of *Giardia* which is commonly known as *Giardia muris.* Most of the material of this order was obtained from culture rats and culture mice of the colony belonging to the Department of Medical Zoology. A few specimens the writer owes to the courtesy of Professor C. A. Kofoid. In this connection, it may be mentioned that an examination of some 30 wild rats and 18 wild mice, secured in Baltimore, showed that none of these animals harbored giardias. Two wild rats and four house mice which were caught near Chester, Nova Scotia, were likewise free.

(*e*) In the course of the present study, two culture rats were found to be infected with two types of *Giardia.* These will be considered in detail later on.

The material was examined partly in the living state, partly after fixation in Schaudinn's solution and after staining with Heidenhain's haemotoxylin, Giemsa's stain, and Mann's stain.

1. A COMPARISON OF THE HUMAN TYPE OF *Giardia* WITH THAT FOUND IN CULTURE RATS AND CULTURE MICE.

At the time when Grassi made his discovery of the occurrence of giardia in *Mus musculus, Mus decumanus,* and *Arvicola arvalis* (1879), he was probably more interested in the general fact of the occurrence of parasites of this order in the animals mentioned, than in the question whether they beonged to the same species as those found in man. The problem of the identity of the human and the rat-mouse form was, as a matter of fact, not raised until 1908, when Benson published his well-known study on the structure and species of the genus *Lamblia.* In this study he pointed out what he considered as essential differences between the forms occurring in man, the mouse, and the rabbit and gave to the mouse form the name of *Lamblia muris.* From the context it is not altogether clear whether he studied this type in wild mice or in culture mice, though the latter is the more probable.

Admissibility of using culture rats for this investigation.—Inasmuch as Grassi found the parasite in question in *wild* rats and mice, it may be questioned whether any conclusions regarding morphological differences based upon the examination of "culture" material would be valid. The writer was particularly anxious to secure naturally infected wild rats and mice, but neither in Baltimore nor Nova Scotia could he find any but "clean" animals. But, as the culture rat is merely a domesticated variety of the Norway rat (*Mus decumanus*), and the culture mouse of the common house mouse (*Mus musculus*), the conclusion seemed justifiable that the type of giardia which is so common in these domesticated strains would be the same as that found in the wild forms or at least that the type occurring in the wild strains could maintain itself in the domesticated strains. As a matter of fact no descriptions of the type found in the wild mouse and rat exist. The probable correctness of the conclusion that the forms are identical is supported by the writer's findings, that *Mus decumanus* can be infected readily with the common giardia of culture mice and culture rats and that no morphological differences develop in the new host (see below).

Necessity of establishing standards for purposes of comparison.—In my paper on the type of giardia occurring in man (1921), I pointed out that for comparative studies it was essential to analyze the descriptions furnished by other investigators, and to attempt to harmonize any conflicting opinions that might have been expressed, so that a standard should become available for a discussion of questions of specificity. The same will be necessary in connection with the animal forms under consideration.

Number of blepharoplasts and axostyles.—The first adequate description of the mouse type was furnished by Wenyon (1907). His studies were based on material obtained from (presumably) culture mice of the Pasteur Institute of Paris and of the Zoological Institute of Munich. His conception of the structure of the organism can be seen at a glance by referring to Plate XVI, Fig. 1. In 1908 came Bensen's description of the organism (Plate XVI, Fig. 2), and in 1915 that of Kofoid and Christiansen (1915b) (Plate XVI, Fig. 3). A study of these descriptions shows the following points on which the writers are not in accord. Both Wenyon and Bensen describe and picture the presence of a pair of granules (blepharoplasts) lying between the two nuclei and a little anterior to the anterior poles of the latter, from which the antero- and postero-lateral flagella originate, and between these of two additional structures, granule-like according to Bensen and rod-like according to Wenyon, from which two axostyles originate and become continuous with the two caudal flagella. But, while Wenyon saw no communicating fibril uniting the outer set of granules to the corresponding nuclei, Bensen pictures a distinct rhizoplast running from each granule to a granule in the anterior border of the corresponding nuclear membrane.

Kofoid and Christiansen in their description of the mouse form state (1915, p. 35) that they found two large, spheroidal blepharoplasts, ''symmetrically placed at the right and left sides of the anterior end of the axostyle'' (which latter they conceive to be single in the resting stage of the animal), ''joined by a slender, deeply staining transverse fibre or commissure. . . .'' They further state that each karyosome is connected with the corresponding blepharoplast by means of a rhizoplast, with the interposition of an ''anterior membrane granule.'' In other words Kofoid and Christiansen do not recognize the existence of the four granules of Wenyon and Bensen, in the resting stage of the animal.

My own findings are in the main in accord with those of Kofoid and Christiansen but I do not recognize the existence of a single axostyle.* The question then arises how to account for the four granules of Wenyon and Bensen. It is possible that these writers encountered forms in which binary division had begun and in which four blepharoplasts actually were present, and that they regarded this as the normal condition. In such an event the writers should theoretically have seen not only the two axostyles which are present in the quiescent stage, but also the beginnings of the second set of axostyles. But it must be borne in mind that at that stage in the development of our knowledge of the structure of these organisms these very fine points may well have been overlooked, and that in the mouse form these details are particularly hard to make out. In my human material I have found forms with dividing blepharoplasts only on rare occasions, while in some of my animal material they were not so uncommon, and in Dr. Hegner's preparations of the dog and rabbit form (1922) they were more numerous than forms with a single pair only. We may conclude that the number of internuclear granules depends upon the stage of development of the organism, that two blepharoplasts only exist during the resting stage of the animal, while four may be encountered at a very early stage of division. The two blepharoplasts are united with each other and with the karyosomes of the corresponding nuclei by an exceedingly fine fibril which in the mouse form can be seen only in exceptionally favorably differentiated specimens. It is in the writer's experience more common in the mouse form to find the terminal portions of the axostyles attenuated rather than clubbed, and apparently not united either with each other or the nuclei (Text figure 3).

Having come to the above conclusion regarding the number of blepharoplasts and their connecting fibrils we cannot agree with Bensen that the mouse form can be distinguished from the human type on the basis of the presence in the latter of a fibril uniting the karyosomes to the blepharoplasts and these to each other.

Basal granules.—Bensen has pictured the presence of a basal gran-

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* In their recent publication on *Giardia enterica* Kofoid and Swezey (1921) state that this form has two axostyles. It is gratifying to the writer to note that Kofoid and Swezey are in agreement with the position of the writer and all other observers on this point. Inasmuch as they had previously insisted that in the giardia of the culture mouse and the California meadow mouse the axostyle is a single organ, we are probably justified in inferring that they have abandoned this idea, and in their table of comparison between rodent forms and the human form they do not refer to such differences, which they no doubt would have done, if they still believed in the single axostyle.

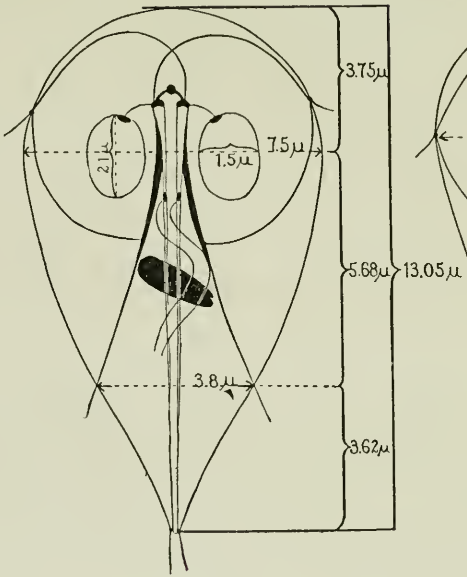

FIG. 1.

FIG. 1. Composite figure of *Giardia enterica*, based on measurements of ten individuals, two each from five different hosts.

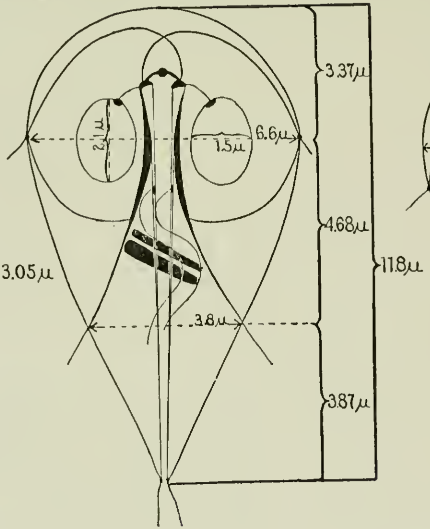

FIG. 2.

FIG. 2. Composite figure of *Giardia microti*, based on measurements of ten individuals, two each from five different Nova Scotia meadow mice.

All measurements are given in micra.

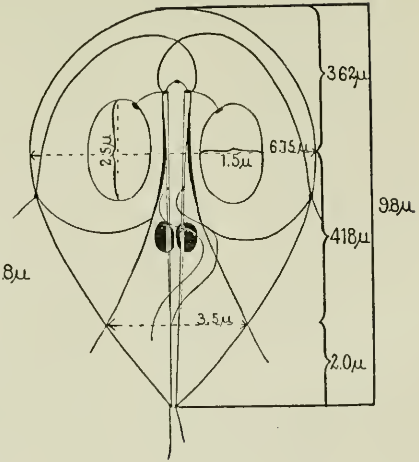

FIG. 3.

FIG. 3. Composite figure of *Giardia muris*, based on measurements of ten individuals, two each from five different hosts.

ule at the points of exit from the body of the parasite of the antero-lateral and caudal flagella, both in the mouse form and the human form, also at the points of exit of the postero-laterals in the mouse form, but not in the human form. Wenyon makes no mention of basal granules of this order in his description of the mouse form, while Kofoid and Christiansen state that there is such a granule where the antero-laterals emerge, none as a rule at the points of exit of the postero-laterals, ''though in some individuals a small one is present.'' Where the caudals leave the body Kofoid and Christiansen state ''there may be a basal granule, but this is not always present.'' ''After the division of the single axostyle to form two, one flagellum and one basal granule go with each daughter axostyle.'' This view the writers no doubt have abandoned. In their paper on *Giardia enterica* Kofoid and Swezey state that there is no distinct basal granule at or near the point of emergence of either the antero-lateral, the postero-lateral or the caudal flagella in that parasite. This is erroneous so far as the postero-lateral and caudal flagella are concerned, each of which starts from a basal granule. The mouse-rat form cannot be differentiated from the human form, however, on the basis of the presence or absence of the granules, as they cannot be distinguished in all specimens. At the points of emergence of the postero-laterals I have not seen structures of this type in either form.

The ''parabasal'' bodies.—In discussing the essential points of difference between the mouse form and the human form, Bensen lays a good deal of stress upon the character of the parabasal body (räthselhafte Körper) or bodies. He thus states that in the human form ''the body in question is very large, located transversely, that it is thicker in the center but does not become much narrower towards its rounded ends.'' Bensen evidently regards it, as also that of the mouse form, as a single organelle. The mouse form he describes as of plump appearance, almost entirely round and not rarely surrounded by a halo (clear zone). Wenyon recognizes the presence of two very dark staining bodies of this order, one on each side of the middle line. Kofoid and Christiansen state that the mouse form is provided with a pair of ''large, subellipsoidal, deeply staining masses, sometimes fused into one, lying dorsal to the middle of the axostyle.''

My own observations on the mouse form agree with those of Wenyon and Kofoid and Christiansen, but I have not found the parabasal either uniformly present or of uniform size. In my human material I have rarely met with appearances which suggest the existence of two parabasal bodies and there also I have found this structure of inconstant occurrence. When present and fully developed it appeared club-shaped which feature might well serve to differentiate the corresponding organism from the mouse form, but unfortunately it is very frequently absent, or imperfectly developed.*

We may conclude then that with the exception of the parabasal body no essential structural differences exist between the mouse form and the human type and that apparent differences are merely the expression of stages in the life history or development of organisms.

Size and form.—If now we turn to the question of size and form we meet with differences which are quite striking. Bensen clearly recognized this. He described the human form as of slender outline, not much wider in the middle than in the anterior portion, while posteriorly the lines run almost straight to the tail. Of the mouse form he says that this attains only three quarters of the size of the human form, that it has a short stocky shape and that the lines of the posterior portion, in converging toward the caudal extremity, are distinctly concave. He does not give any actual measurements, however. Inasmuch as several later observers (Noc 1909) state that size cannot serve as a criterion of differentiation, without, however, giving actual data, it seemed indicated to the writer that this factor should be studied in detail.

To this end 250 specimens of the mouse form, representing five different hosts, were measured in reference to length and breadth of body, from which the ratio L/B was calculated. The minimum, maximum and mean values for each set of 50 are shown in Table I, as well as the composite values, with the probable error of the mean.

In the same table the corresponding values for the human form are given. In chart 1 will be found the frequency values for the two forms, as well as the type which occurs in the meadow mouse, the general morphological comparisons of which with the human form will be considered below.

A study of the tabulated values will show that there is a marked difference in the length of the two types of organisms. A comparison of the composite means shows a difference of 3.95 with a corre-

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* In a recent case of human giardiasis which occurred under the observation of Dr. Walter Thomas of the Clifton Springs Sanitarium and in which living material was obtained directly from the duodenum, the writer met with fairly numerous specimens in which two distinct parabasal bodies could be discerned. In fecal material such pictures are however very rare.

TABLE I.

Measurements in micra of the trophozoites of Giardia muris and Giardia enterica.

These measurements have reference to the trophozoites from five different cases of infection, with *Giardia muris* and *Giardia enterica,* respectively.

	Number of specimens.	Length.			Breadth.			Ratio of length to breadth.		
		Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.
Mouse Form:										
K..........	50	7.75	12.25	9.44	5.25	8.25	6.82	1.1	1.7	1.39
B and W...	50	8.25	11.75	10.36	6.75	8.75	7.56	1.1	1.5	1.38
B..........	50	8.75	12.75	10.35	6.75	9.75	8.08	1.1	1.4	1.29
Y..........	50	8.25	11.25	9.83	6.25	8.75	7.18	1.1	1.6	1.37
R.M........	50	7.25	11.25	8.77	5.25	8.25	6.67	1.1	1.6	1.32
Composite....	250	7.25	12.75	9.75‡ ±0.04	5.25	9.75	7.26‡ ±0.04	1.1	1.7	1.35‡ ±0.006
Human Form:										
Maryland I	65	10.25	20.25	14.11	6.25	10.25	7.87	1.4	2.2	1.80
Maryland II.......	82	11.25	17.25	14.34	6.25	9.25	7.83	1.5	2.3	1.80
Maryland III......	50	9.25	15.25	11.61	5.00	8.00	6.31	1.5	2.0	1.79
South Carolina.....	73	10.25	15.25	12.70	5.75	8.75	6.94	1.4	2.2	1.80
French.....	118	11.25	17.75	14.28	6.75	10.25	7.82	1.4	2.3	1.60
Composite....	338	9.25	20.25	13.70‡ ±0.2	5.00	10.25	7.46‡ ±0.034	1.4	2.3	1.84‡ ±0.009
Differences between the two forms..		0.00	7.50	3.95	0.25	0.50	0.20	0.3	0.6	0.49

sponding probable error amounting to 0.3031. This of course is highly suggestive. But it might be argued that the two types of organisms might after all be the same, and that the difference in size is merely the expression of a difference in environment. This, how-

‡ The composite means were obtained, not from the means of the five animals, but from the measurements of the total number.

ever, is improbable. Differences in environment might possibly affect the mean length, but it would hardly bring about such a widely different type of distribution as we have in the present instance and as is shown in chart 1.

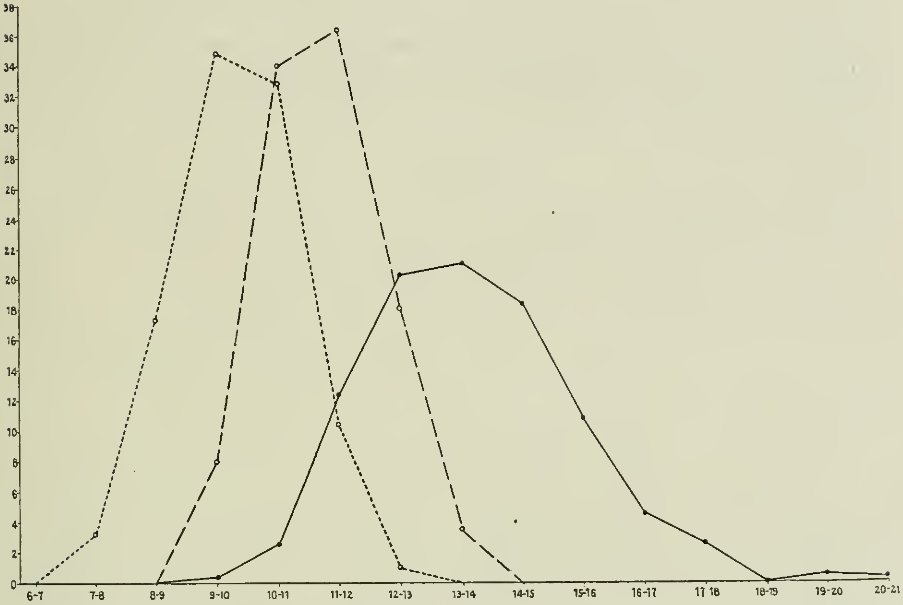

CHART 1. Percentage distribution values for the measurements of length of 250 specimens of *Giardia muris* (dotted line), 250 specimens of *Giardia microti* (broken line), and 388 specimens of *Giardia enterica* (solid line).

TABLE II.

Showing measurements of the mouse type found in a white mouse and in its subsequent host—a black and white rat.

	Length of body.			Breadth of body.			Ratio of length to breadth.		
	Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.
Mouse.	8.25	11.25	9.83	6.25	8.75	7.18	1.1	1.6	1.37
Rat.	7.25	11.25	8.77	5.25	8.25	6.67	1.1	1.6	1.32
Differences.	1.00	0.00	1.06	1.00	0.50	0.51	0.0	0.0	0.05

The probable correctness of this view is strengthened by the fact that a transfer of a strain occurring in a white mouse to a white rat (by feeding) resulted in insignificant differences (Table II) only and hardly changed the curve of distribution at all (see chart 1) and last, but not least, we encountered a double infection in a culture rat, where an evident mouse form lived together with an entirely different type. The findings in this animal will be found tabulated in Table III and the length and length/breadth frequency distribution in charts 5 and 6. The differences are so marked that they require no special comment.

TABLE III.

Showing measurements which were obtained in a coinfection in a culture rat, one type being the common mouse-rat type, the other that which the writer has temporarily termed Giardia sp.

	Length of body.			Breadth of body.			Ratio of length to breadth.		
	Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.
G. muris...........	8.75	12.25	10.35	6.75	9.75	8.08	1.1	1.4	1.29
G. sp..............	10.25	16.75	13.25	6.25	9.25	7.49	1.4	2.1	1.78
Differences.........	1.50	4.50	2.90	0.50	0.50	0.59	0.3	0.7	0.49

Of the origin of the large form in this case, I have no knowledge. The animal was a member of the rat colony of the Department of Medical Zoology and had not come in contact with other animals either directly or indirectly for a period of at least six months, so that we may assume that the two types of organisms lived side by side in the same animal for at least that length of time.

In going over Boeck's paper entitled "Studies on *Giardia microti*" (1919), I find that this investigator encountered a type of giardia in culture rats in Berkeley, California, which "resembled *G. microti* as far as the general form of the body and the organelles are concerned." It measured from 13 to 17 μ in length and from 6 to 9 in width. I am inclined to think that we were both dealing with the same type, but I do not believe that we can regard this as *Giardia microti* in view of the fact that in my experience culture rats cannot be infected with that type (see experimental data below).

In principle the observation of this coinfection is of course of the greatest importance and strongly supports the probability of the existence of different species.

A comparative study of the breadth values of the mouse and the human type (Table I and chart 2) does not reveal such clear-cut differences, either in the ranges or the means, nor in the type of distribution, as were shown by the length values. An application of the χ^2 test for "goodness of fit" gave a value of 3.16 for χ^2 and of 0.532 for P (probability that the differences obtained between the frequency distributions of the two forms are due to errors of random sampling), which means that 53 times out of 100 one would expect

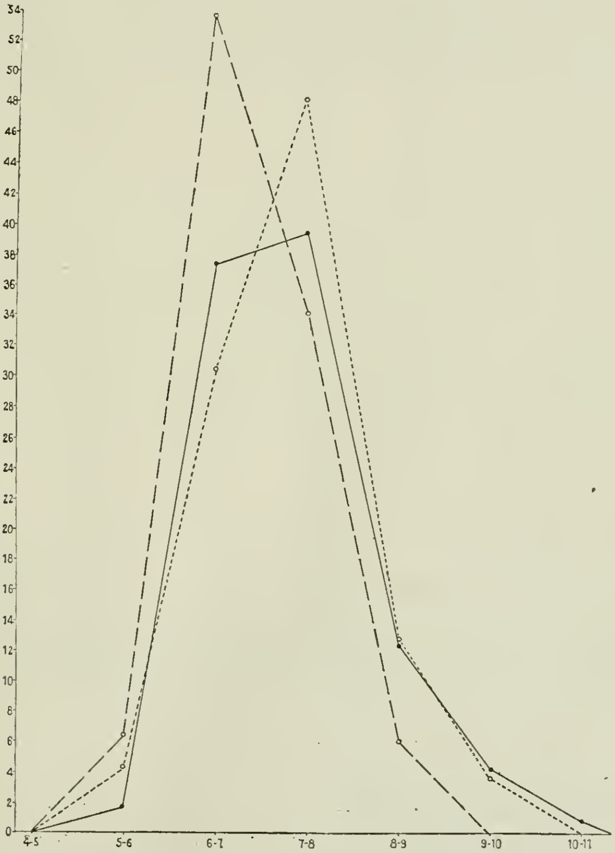

CHART 2. Percentage distribution values for the measurements of breadth of 250 specimens of *Giardia muris* (dotted line), 250 specimens of *Giardia microti* (broken line), and 388 specimens of *Giardia enterica* (solid line).

as great or greater divergences due to errors of random sampling than are shown in the table. We accordingly conclude that on the basis of breadth the existence of different species cannot be proven in the case of the mouse and the human type, but we would emphasize that this does not mitigate against the conclusions reached on the basis of length that they are different.

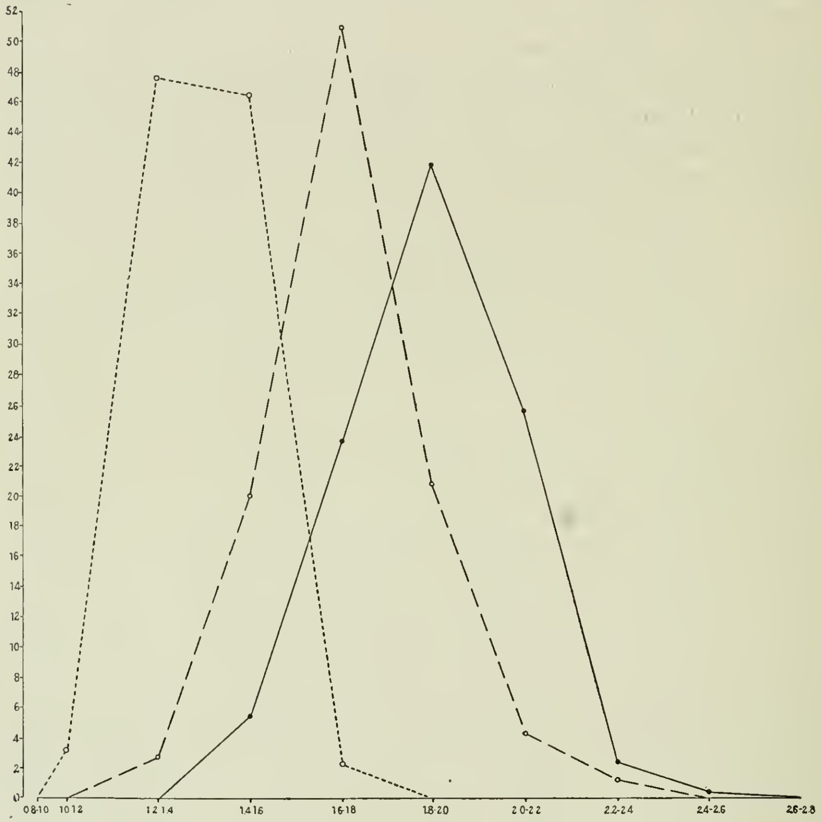

CHART 3. Percentage distribution values for the measurements of length divided by breadth of 250 specimens of *Giardia muris* (dotted line), 250 specimens of *Giardia microti* (broken line), and 388 specimens of *Giardia enterica* (solid line).

The values which were obtained for the rates of length and breadth (L/B) reveal an entirely different size ratio between the human and mouse type and also a somewhat different type of distribution. But, inasmuch as the breadth values differ but little, while the length values differ very widely, it is clear that these differences in ratio of length to breadth are merely the expression of the difference in length and need not be discussed further. (Table I and Chart 3).

A comparative study of the cysts of the human and the mouse type.—Our study of the dimensions of the cysts of the two types of organisms under consideration are based upon measurements of 250 specimens of the human type, taken from five different individuals and 100 specimens of the mouse type, taken from two different individuals (both culture rats).

Our results are shown in Table IV and chart 4. They clearly indicate that the two types can hardly be distinguished, the one from the other, on the basis of size. Considering the smaller length of the mouse trophozoite, as compared with the human form, we had ex-

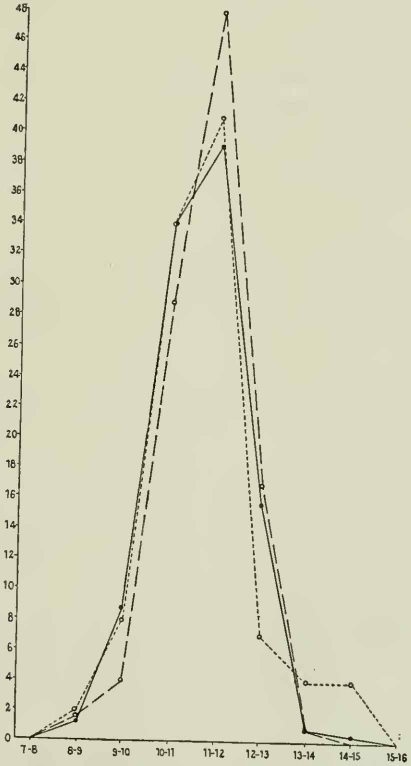

Chart 4. Percentage distribution values for the measurements of length of 100 cysts of *Giardia muris* (dotted line), 250 cysts of *Giardia microti* (broken line), and 250 cysts of *Giardia enterica* (solid line).

pected to find a corresponding difference in the length of the cysts. But the proportionately greater breadth of the mouse form evidently makes a correspondingly large demand upon space, so that in the end the size of the cysts is nearly the same.

TABLE IV.

Showing the size of 100 specimens of mouse cysts, taken from two different individuals, with their composite values, as compared with the corresponding values obtained from 100 human cysts, also representing two individuals.

	Length.			Breadth.			Ratio of length to breadth.		
	Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.
Mouse Form:									
1	8.25	14.25	11.20	6.75	9.25	7.85	1.0	2.1	1.46
2	8.25	13.25	10.86	6.25	8.25	7.29	1.1	2.0	1.51
Composite	8.25	14.25	11.03	6.25	9.25	7.57	1.0	2.0	1.48
Human Form:									
Md. I	8.0	13.0	10.6	6.0	8.5	7.5	1.0	1.8	1.41
Md. III	9.0	12.5	11.0	6.5	10.0	8.2	1.1	1.8	1.33
Composite	8.0	13.0	10.79	6.0	10.0	7.85	1.0	1.8	1.37
Differences of composites	0.25	1.25	0.24	0.25	0.75	0.28	0.0	0.2	0.11

While the two types of cysts can thus not be differentiated from one another on the basis of size they can be readily differentiated morphologically, the essential factor being the appearance of coarse chromidial masses in the cytoplasm of the mouse type, which are either never seen in the human type, or if something suggestive of these masses appears, there is so little of it that it would scarcely attract attention. The multiple loops of the human type which the writer in agreement with Rodenwaldt views as peristomal fibrils are in the mouse type less distinct; if visible at all, appearently single loops only are seen. The longer chromatic rods of the human type which the writer has tentatively viewed as shield border fibrils do not seem to occur in the mouse form and parabasal bodies are apparently absent, as in the human type.*

Feeding experiments.—While our morphological findings in the mouse and the human type of *Giardia* support the idea that the two represent separate species, it occurred to the writer to test this question also by means of feeding experiments.

Unfortunately it has not been possible to secure human subjects for these experiments. It may be mentioned, however, that one attempt to bring about human infection by starting with mouse material was actually made many years ago, by Moritz and Hölzl (1892). These investigators fed cysts from mice to a human being—a tuberculous patient—but did not succeed in bringing about infection. Their reason for choosing a tuberculous subject was the belief that organisms of this order would be more apt to secure a foothold in a diseased rather than in a healthy subject.

Since human subjects were not available for our purposes it occurred to the writer that the experiment might be reversed and still throw light on the question under discussion. It seemed reasonable to suppose that if the human form were identical with the mouse form, and differences in size were merely due to differences in environment, then by feeding human cysts to clean hosts of the original type, the mouse form should again appear in these.

Attempts to infect mice with human material have been recorded by several investigators, viz., Perroncito (1888), Bohne and v. Prowazek (1908), Fantham and Porter (1916), Porter (1920), and Deschiens (1921). The purpose of experiments of this order on the part of the investigators named was, however, not the same as that of the writer. They were interested in the question of the pathogenicity of giardia, and not in that of the origin of the human infection. Inasmuch as the significance of any results reached by experiments of this order will of course depend upon the question whether the animals fed were clean at the time, and not exposed to infection after being fed until the conclusion of the experiment, any experiments in which this factor was not considered may be eliminated from the discussion. This will probably rule out the results which were obtained by Perroncito, as well as those of Bohne and v. Prowazek, for it was not known at the time when these investigators made their studies that the elimination of giardia cysts, both of human and animal origin, could be intermittent and that the intermissions were often of considerable length (Porter, Dobell, Boeck). It may be mentioned, however, that Perroncito thought that he had succeeded in infecting two white mice out of four, while Bohne and v. Prowazek who fed cysts to rabbits, rats, and young cats and at the same time introduced vegetative forms per anum, obtained negative results only.

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* It should be pointed out in this connection that Kofoid and Christiansen (1915) seem to share the writer's belief that there are no parabasals in the cysts of the mouse form, for they state (p. 40): ''It is also absent in encysted stages (pl. 7, figs. 34–43). Its disappearance (in the vegetative forms?) may therefore be incidental to the process of encystment and be correlated with changing conditions of metabolism.'' In their recent publication on *Giardia enterica* (1921), on the other hand, Kofoid and Swezey maintain that parabasals are present in the cysts of this form. They state ''the pairs of curved parabasals are especially prominent'' and ''the increased size of the deeply staining parabasals . . . in the confinement of the cysts. . . .'' A study of their illustrations reveals that they view the deeply staining curved fibrils which Rodenwaldt and the writer regard as peristomal fibrils as dividing parabasals. In the part of their paper on *Giardia muris*, however, in which parabasals were said to be absent, they clearly picture such curved fibrils (Figs. 35 and 38). Evidently their view that parabasals do not occur in the mouse cysts, or that the fibrils in question in the human cysts are parabasals, is erroneous. In the writer's opinion they are mistaken in viewing the fibrils in the human form as parabasals.

Fantham and Porter (1916), on the other hand, state specifically that their animals—kittens and mice—were examined for some time (up to a month) before being fed and that aperients were even used at times to insure that the animals were actually free from a giardia infection. So far as the results from their experiments on mice are concerned (these being the ones that interest us more especially in this connection), it appears that of the nine animals used seven developed the infection and of these four died, while the other two proved resistant. They conclude that mice may serve as reservoirs of infection for human beings.

Very remarkable are the results which Porter obtained in South Africa. This writer states that she found giardias in samples of water "used previously by the parents of native children at the Johannesburg hospital in preparing the food for the infants, identical with those present in the motions of the children." Clean rats whose food was mixed with this contaminated water contracted flagellate diarrhoea and passed large numbers of parasites in the feces.

Deschiens reports experiments on cats and mice. Only the latter concern us at this place. He states that his animals were examined daily for two weeks preceding the feeding experiments. He found that in four animals out of five the ingestion of cysts of the human form was followed by a grave or fatal infection in animals which had previously not been parasitized.

My own experiments led to conclusions which are diametrically opposed to those reached by the last three investigators. They were performed on five adult culture rats, and five wild rats. Three of the former had been observed for several weeks in the laboratory and were regarded as clean, while preliminary experiments (by dissection) had shown that our Baltimore city rats are not infected with giardias. The human cysts after being washed by centrifugation with repeated changes of water, until all fecal odor had disappeared, were fed to the animals, partly suspended in their drinking water (inverted bottle arrangement), partly in milk and partly mixed with giardia-free entrails of other rats. Two of the culture rats remained negative for several weeks, so far as the results of fecal examinations went; after this the observations were discontinued. The other animals were killed after intervals varying from seven days to a couple of months and carefully examined for vegetative forms throughout the intestinal tract—all with negative results. It may be mentioned moreover, that none of the animals showed any signs of sickness, viz., diarrhoea.

It might be argued of course that inasmuch as we used culture rats and wild rats the negative results may have been due to our selection of these experimental animals instead of white mice.

To ascertain whether wild rats and culture rats could be infected with the mouse form, the entrails of a white mouse harboring large numbers of giardias of the mouse type were fed to a "clean" white rat. The rat was killed three weeks later and an extensive infection encountered, involving the entire length of the small intestine. The entrails of this animal were then fed to two wild rats. One of these was killed ten days later, the other a month later; both were extensively infected.

We conclude that inasmuch as the mouse form will adapt itself very readily to the intestinal tract of wild rats or culture rats, the human type would likewise have done so, if it were identical with this form.

Since there was evidently no return of the human form to the mouse form in our animals, and after it had been ascertained that two different types of giardias could exist side by side in culture rats, additional feeding experiments with human material were undertaken, using animals which were known to harbor the mouse form, in order to determine what became of the ingested cysts. To this end 6 half-grown "hooded" rats were fed freshly passed, well-washed human cysts (76,400,000) suspended in about 10 c.c. of milk, after the animals had had neither food nor drink for about twenty-four hours. In some instances another feeding of an equal quantity was given twenty-four hours later. The animals were killed after intervals varying from two hours and a half to forty-eight hours. Fresh mounts, as well as Schaudinn specimens, were made from the stomach, duodenum, ileum, caecum, and rectum, and carefully examined for cysts and "large" vegetable forms. In the small intestine small mouse type trophozoites only were found, and in the rectum mouse type cysts in the usual small number, showing the characteristic chromidial masses. I would emphasize particularly that the examination made two hours and a half following the administration of such a large dose of cysts revealed a small number only in the stomach which was filled with milk curds. They were evidently dead, however, for they had lost their characteristic refractivity and stained with eosin. No cysts were encountered below the pylorus.

We may conclude that human giardia cysts when fed to culture rats, both young and old, as well as wild rats, both young and old, are destroyed by the gastric juice of these animals. Our feeding experiments thus support the conclusion at which we had arrived on morphological grounds, that *human giardiasis is not of rodent origin, so far as the mouse type is concerned; that the mouse type, as well as the human type, constitute separate species.* For the former we accept the specific name given by Bensen, viz., *Giardia muris.* The latter was described in our first paper under the name *Giardia enterica,* as suggested by Kofoid and Christiansen. This, however, is untenable according to Stiles and should be replaced by the term *Giardia lamblia,* which also was suggested by Kofoid and Christiansen, though as the result of a misunderstanding of the expression *Giardia (s) lamblia* used by Stiles, in a letter to the former. Kofoid apparently overlooked the *s* and thought that Stiles had actually proposed this name. The name, however, must stand and is perhaps a proper tribute to Lambl who first described and pictured the organism.

II. A COMPARISON OF THE HUMAN TYPE OF *Giardia* WITH THAT FOUND IN THE MEADOW MOUSE.

Probable identity of Kofoid and Christiansen's Giardia microti and the form found by Grassi in Arvicola arvalis.

Until Kofoid and Christiansen (1915a) reported the presence of a form of *Giardia* in the California meadow mouse *Microtus californicus californicus* (Peale), which they regarded as a new species, "differing in appearance, proportions, and stainability from the widely prevalent *Giardia muris*, and not possessing the characteristics of any known species of the genus," it had been an open question whether the organism which Grassi found in *Arvicola arvalis* was identical with the form which the same writer encountered in rats and mice.

During the summer of 1920 I found in the common meadow mouse of Nova Scotia, which was identified as *Microtus pennsylvanicus acadicus*, a giardia which, excepting for slight differences in size, is apparently identical with that described by Kofoid and Christiansen. Structurally I have been unable to note any essential differences, although here also I cannot follow Kofoid and Christiansen in accepting the existence of a single axostyle.* As in the human form I found a granule located in the center of the interblepharoplastic commissure, which Kofoid and Christiansen do not mention nor picture, but which I was able to demonstrate also in specimens of the California type kindly loaned me by Dr. William C. Boeck.† During the past autumn (1921) I have further encountered the same or a very similar species in the common meadow mouse of Maryland, which indicates sufficiently the probably uniform distribution of this type over the North American continent.

In the same year (1920) that I discovered the occurrence of this type of giardia in the meadow mice of Nova Scotia, there appeared in the Annali d'Igiene an account by Splendore (1920) of the parasites of "Arvicola" in Italy.* The arvicola in question was diagnosed as *Pitymys Savii Selys,* which, so far as the writer knows, does not occur in North America, but which, to judge from an accompanying illustration, closely resembles the Microtinae. Splendore states that this species is very common in southern Italy. Whether it occurs in Lombardy, where Grassi made his initial observations, I do not know, but it would seem likely that this is the animal to which he referred by the name of *Arvicola arvalis.* In this species Splendore encountered a giardia which he has described under the name *Giardia pitymysi,* n. sp. Unfortunately Splendore has given no measurements of the organism, merely stating that its size is variable, but he has furnished a good description, accompanied by illustrations (Plate XVI, Figs. 5 and 6) which are sufficiently detailed to make it clear that he was not dealing with the mouse form which we have considered above. So far as one can judge the organism corresponds to *Giardia microti.* He pictures two axostyles and four basal granules like Wenyon and Bensen, and in addition a fifth granule situated on the arched commissure uniting the central pair of granules, which clearly corresponds to the interblepharoplastic granule of the writer.† Since Splendore shows a rhizoplast connecting each outer granule with a granule located in the anterior portion of the nuclear membrane, and the latter with the karyosome, he cannot have confused his outer granules with the centrosomes, and I can only conclude that he also, like Bensen and Wenyon, observed forms in which division of the blepharoplasts had already taken place. In two of the three illustrations parabasals are represented and absent in one. They are double, resembling two parallel commas, which obliquely cross the long axis of the animal. The appearance of the parabasals, in association with the elongate form of the organism, clearly separates this type from the mouse type.

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* But inasmuch as Kofoid acknowledges the existence of two axostyles in his recent paper, dealing with the human type of *Giardia*, I dare say that he has abandoned his former position in reference to *Giardia microti*, and would now be willing to concede two axostyles also in this organism.

† It is possible that Kofoid and Christiansen, under the influence of their doctrine of the existence of a single axostyle misinterpreted the interblepharoplastic granule as constituting their hypothetical second pair of blepharoplasts, in supposedly dividing forms. So much is certain that forms with four blepharoplasts are rarely seen, and when dividing forms do occur the second pair lie posterior to the original pair and not between the two.

The likelihood that the meadow mouse is responsible for the human infection is, of course, on *a priori* grounds, even smaller than that the wild mouse or the wild rat represents its source, for bearing in mind that giardiasis is quite common amongst city dwellers, children especially, it would be difficult to conceive how these could become infected by a rodent which can hardly be regarded as a city dweller itself. Amongst country people, on the other hand, such a possibility would not seem so remote, and if we compare the meadow mouse form with the human form there is certainly a striking resemblance, and one cannot help but feel that even though the two may *now* constitute different species, some time in the dim distant past man may have acquired his infection from this source. But the problem before us is to decide whether the two forms are identical at the present time, or whether they represent separate species.

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* Deschien's (1921) statement that the human type of *Giardia* was observed in Italy in *Mus musculus, M. rattus, M. decumanus, M. sylvestris,* in the dog, the cat, the sheep, and the rabbit is incorrect. It is evidently based on Grassi's earlier statement regarding the occurrence of giardias in these animals, which was made at a time when the question of the existence of species had not yet been raised.

† I note that Kofoid and Swezey interpret this arched commissure as sprouting flagella, but I cannot share this view. I hope to enter into a discussion of some of their differing interpretations on another occasion. I have already pointed out that a double commissure exists when a double set of anterior flagella can be seen, set for set.

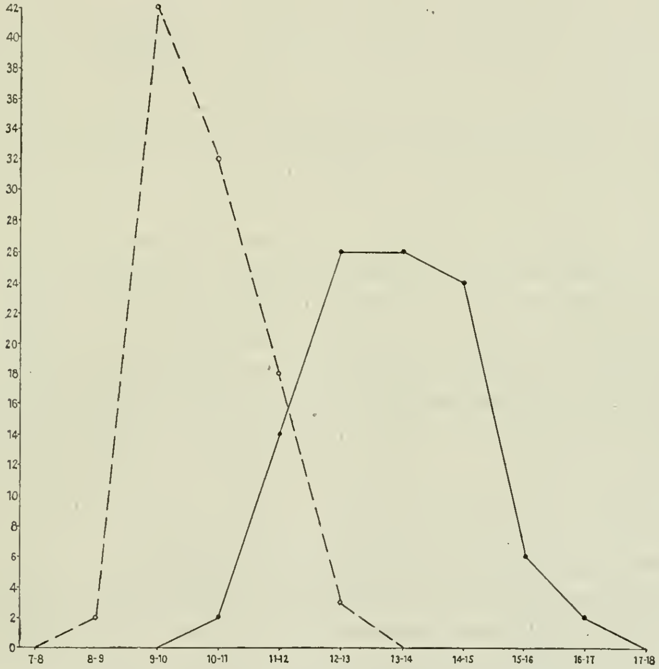

CHART 5. Percentage distribution values for the measurements of length of 50 specimens of *Giardia muris* (broken line) and 50 specimens of *Giardia sp.* (solid line).

So far as the general morphology of the two forms is concerned no essential points of difference seem to exist excepting in the form of the parabasals and the size of the trophozoites (Text figures 1 and

2). As in the human form and the mouse form the parabasals are frequently absent and I do not hesitate to say that where this is the case it is impossible to differentiate between the two, excepting on the basis of size (see below).

In their paper on the meadow mouse form Kofoid and Christiansen state that there is a "marked difference in the parabasals, which are slender and curved in *G. microti,* but much larger and massive and apparently fused in one body, according to Bensen (1908), in the human form." In referring to Rodenwadlt's figures, however, they find that these leave "little doubt that there are, at times, at least, two stout brush-shaped parabasals in an obliquely curved position (in the human form) . . . ." "These are very much like those of *G. microti* except that they are a little stouter." *

My own observations accord with those of Kofoid and Christiansen that in the meadow mouse form two parabasals can often be distinguished, when present at all. Kofoid and Swezey (1922) state that in the human form also there are two parabasals, and they point out that I have erroneously represented the parabasals as single, in my drawing of the human form in Hegner and Cort's Diagnosis (1921).

In my detailed description of the human form (1921) I stated that the parabasal body usually presents a club-shaped or a heavy comma-like appearance, and that occasionally one gets the impression as though the structure were split longitudinally. If Kofoid and Swezey were correct in their statement that "typically" the parabasals appear as a pair of comma-shaped structures it should be unnecessary to add that "failure to focus out and analyze the pair of parallel parabasals underlies the interpretation . . . of these organs as a single rather than a paired structure" and that they "find it possible to analyze the seemingly single bodies into the pair . . . ."

In my experience, the double or split appearance in the human form is the exception and not the rule, and this fact constitutes an actual point of difference between the two forms. Other investigators evidently share this view.

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* In this connection I would point out that Boeck (1917), one of Kofoid's pupils, makes the following statement (p. 8) in reference to the parabasals of *Giardia microti:* "The parabasal bodies composed of one broad or two narrow bands . . . which in some cases appear fused in one body. . . . It plays no part in mitotic activity." In his plate of Figures 1, 2, 3, 4, 9 and 11 he represents it as single.

In their brief discussion of the "specific differences" between the human form and *Giardia* from rodents, which is embodied in their paper on Mitosis and Fission in *Giardia enterica* (1922) Kofoid and Swezey refer to the shape of the blepharoplasts and state that in the human form the head of the axostyle is not expanded by them, while in the meadow mouse form it is expanded. Inasmuch as Kofoid has not yet formally abrogated his claim to a single axostyle in the latter form, but accepts two axostyles for the human form, this is somewhat confusing. I have already emphasized that no differences exist between the two forms in this respect.

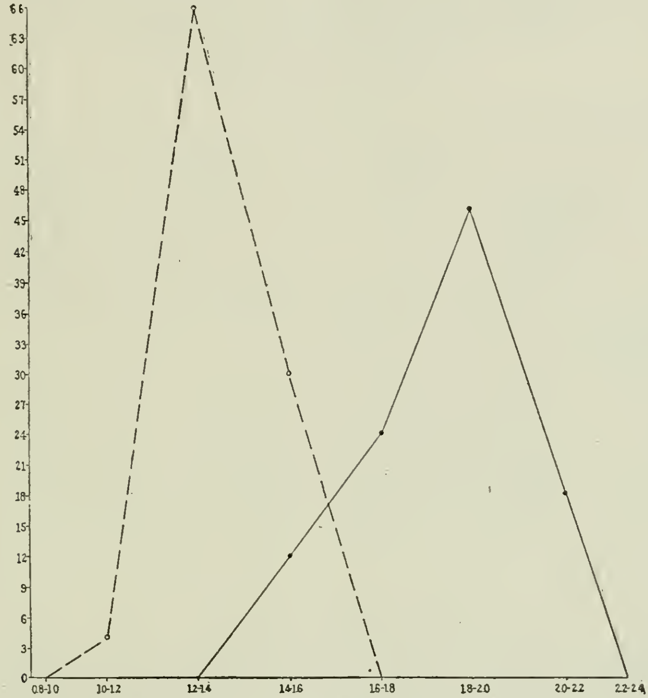

Chart 6. Percentage distribution values for the measurements of length divided by breadth of 50 specimens of *Giardia muris* (broken line) and 50 specimens of *Giardia sp.* (solid line).

Kofoid and Swezey further suggest that in the meadow mouse form there is an antero-lateral and a posterior basal granule while in the human form these are absent. In my experience, based upon the examination of many hundreds of specimens, these granules were

TABLE V.

Measurements which were obtained in the study of the body of the trophozoite from five different cases of Giardia microti infection (Nova Scotia type), together with the corresponding composite values, as compared with the figures which were obtained from five different cases of Giardia enterica infection and their corresponding composite values.

	Number of specimens.	Length of body.			Breadth of body.			Ratio of body length to breadth.		
		Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.
Meadow mouse										
16	50	9.25	13.25	11.18	5.75	8.25	7.09	1.3	2.0	1.61
13	50	9.95	12.75	11.10	5.75	8.25	7.01	1.3	2.0	1.62
12	50	9.25	12.75	11.01	5.25	8.75	6.66	1.3	2.2	1.68
2	50	9.25	13.25	10.71	5.25	7.25	6.33	1.3	2.2	1.74
22	50	9.25	13.25	11.25	5.25	7.75	6.86	1.4	1.9	1.65
Composite‡	250	9.25	13.25	11.06 ±0.04	5.25	8.75	6.79 ±0.03	1.3	2.2	1.66 ±0.008
Maryland I.....	65	10.25	20.25	14.11	6.25	10.25	7.87	1.4	2.2	1.80
Maryland II....	82	11.25	17.25	14.34	6.25	9.25	7.83	1.5	2.3	1.80
Maryland III...	50	9.25	15.25	11.61	5.00	8.00	6.31	1.5	2.0	1.79
South Carolina .	73	10.25	15.25	12.70	5.75	8.75	6.94	1.4	2.2	1.80
French........	118	11.25	17.75	14.28	6.75	10.25	7.82	1.4	2.3	1.60
Composite*....	388	9.25	20.25	13.70 ±0.2	5.00	10.25	7.46 ±0.034	1.4	2.3	1.84 ±0.009
Difference between the two		0.00	7.00	2.64	0.25	1.50	0.67	0.1	0.1	0.18

as frequently found to be present in the human form as in the animal forms, and as frequently absent in the animal forms (both rats, mice, and meadow mice) as in the former. Their presence or absence certainly does not constitute a specific difference. Like Kofoid and his

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[*] The composite means were obtained, not from the means of the five animals, but from the measurements of the total number.

collaborators, I have found no basal granule at the points of exit of the postero-lateral flagella in any form.

As regards general differences in form my findings agree with those of Kofoid and Swezey, viz., that the human form tends to a concave outline along the lateral contour of the tail, while the meadow mouse form is more nearly straight, though this difference is not pronounced in many specimens.

The most important difference between the two forms is that of size and on size alone in my opinion the two forms can be differentiated, providing that a sufficiently large number of specimens be examined. My findings in regard to variations in size are given in Table V and charts 1, 2 and 3.

It will be noted that there is not only a material difference in mean length, but also an entirely different type of distribution. The differences in mean breadth are not so marked, but there is a considerable dissimilarity in distribution here also, which is further brought out by the χ^2 test.

The value for χ^2 was found to be 11.5471 and for P, 0.0009, which means that only 9 times in 1,000 would we expect as great or greater divergences due to errors of random sampling. While this divergence is much more striking than that between *G. muris* and *Giardia enterica* too much stress should not be layed upon this difference.

TABLE VI.

A comparison of the measurements, in micra, of 250 cysts of Giardia microti (composite material from a colony of 20 animals) with 250 cysts of Giardia enterica (composite values from 5 cases).

		Length.			Breadth.			Ratio of length to breadth.		
		Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.
Meadow mouse form	250	8.25	13.75	11.11	5.25	10.25	7.58	1.1	1.9	1.49
Human form.	250	8.00	14.00	10.70	6.00	10.00	7.47	1.0	2.0	1.37
Difference...		0.25	0.25	0.41	0.75	0.25	0.11	0.0	0.1	0.12

The differences in the ratio between length and breadth between the two forms are equally striking.

A comparison of the cysts of Giardia enterica with those of Giardia microti.—In this connection I would briefly point out that, while no essential points of difference exist in the size of the cysts of the meadow mouse form, as compared with the human form (see Table VI and chart 4), it is noteworthy that the cysts which appear in the feces of man never show any evidence of plasmotomy and have for the most part not progressed beyond the quadrinucleate stage, with the retention of the second pair of nuclei close to the first pair. A large percentage of the meadow mouse cysts, on the other hand, as they appear in the feces, contain two perfectly formed individuals. This alone would serve to differentiate the two forms.

Feeding experiments.—In conclusion I would refer to a number of experiments in which the attempt was made to infect wild rats, culture rats, and wild mice with the meadow mouse giardia. In all but one of these experiments the entire entrails of freshly killed, naturally infected meadow mice were fed to wild rats and culture rats. The animals were killed after a number of days and the intestinal contents from various sections examined for the presence of giardias of the meadow mouse type. The results were always entirely negative. In the one exceptional instance, four young house mice, just able to eat alone, were taken from a nest (in the seat of an old automobile) and placed in a large cage, containing twenty naturally infected meadow mice, where they lived together, under ground (sod), for three months. A fifth mouse of the same litter was killed and examined for protozoa with a negative result. During the three months the young mice were never seen to feed in the day time, when the meadow mice invariably appeared, and were obliged to drink heavily cyst-infected water, which was left by the latter, during a period of almost six weeks, while the animals were fed no fresh vegetables. The earth was heavily infected and the mice thus freely exposed. At the expiration of the three months but one of the house mice could be examined (the rest escaped), and although a careful search was made throughout the intestinal tract no giardias of any kind were found, whereas nearly 100 per cent. of my meadow mouse material was found infected. Evidently, it is just as impossible to infect house mice with the giardia of meadow mice as it was found impossible to infect wild rats and culture rats with the human form.

SUMMARY.

1. On the basis of our morphological, biometrical and experimental studies we believe to have established that specific differences exist between the human giardia and the mouse form, as was first suggested by Bensen and subsequently by Kofoid and Christiansen, and that similar differences also exist between the human form and the meadow mouse form and between the latter and the mouse form, as was first suggested by Kofoid and Christiansen, though in both instances on what we regard as insufficient evidence.

2. It seems warrantable to assign species names to the three forms, and we accept that of *muris* for the mouse form, as suggested by Bensen, and the name *microti* for the meadow mouse form, as suggested by Kofoid and Christiansen. For the human form we believe that Stiles, as set forth above, is justified in rejecting Kofoid's term *enterica* and that according to the rules of nomenclature the organism should henceforth be known as *Giardia lamblia.*

3. Culture rats and wild rats cannot be infected with *Giardia lamblia,* while they may readily be infected with *Giardia muris.*

4. Culture rats, wild rats, and wild mice cannot be infected with *Giardia microti.*

5. There is no basis for the assumption that the human infection is referable to either rats, mice, or meadow mice.

6. We believe that the human infection is of human origin.

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EXPLANATION OF PLATE XVI.

FIG. 1. Reproduction of Wenyon's representation of the giardia found by him in culture mice (1907).

FIG. 2. Reproduction of Bensen's figure of *Giardia* (*lamblia*) *muris* (1908).

FIG. 3. Reproduction of Kofoid and Christiansen's representation of *Giardia muris* (Pl. 5, Fig. 1) with quiescent nucleus (1915).

FIG. 4. Reproduction of Boeck's representation of *Giardia microti* in resting stage (Pl. 1, Fig. 1, 1917).

FIGS. 5 and 6. Representations of Splendore's Figs. 3 and 4 (Pl. 7) of *Giardia pitymysi* (1920).

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PLATE XVI.

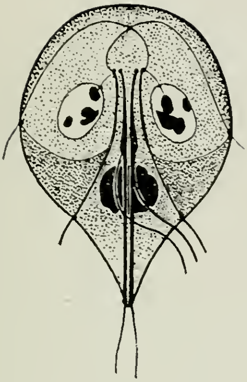

Fig. 2.

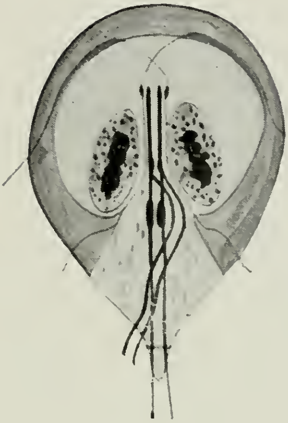

Fig. 1.

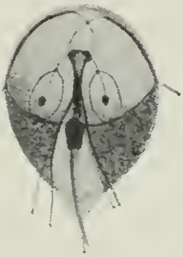

Fig. 3.

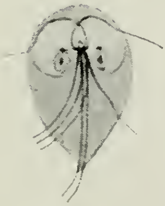

Fig. 5.

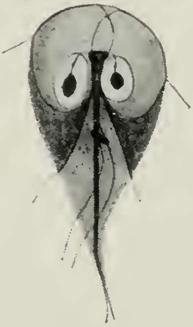

Fig. 4.

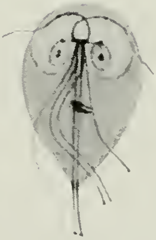

Fig. 6.

THE SYSTEMATIC RELATIONSHIP OF GIARDIA LAMBLIA STILES, 1915, FROM MAN AND GIARDIA AGILIS KUNSTLER, 1882, FROM THE TADPOLE.*

By ROBERT W. HEGNER, Ph.D.

(Received for publication, April 24, 1922.)

1. The objects of this contribution are (1) to furnish a modern description with figures and measurements of *Giardia agilis* from the tadpole, which may be used (2) to determine the systematic relationship of this species and *Giardi lamblia* from man, and (3) to record certain variations in the size of specimens of *G. agilis* from different tadpole hosts.

2. Material and methods.—Material from 34 tadpoles of the green frog, *Rana clamitans*, was obtained from Chester, Nova Scotia, through the kindness of my colleague, Dr. Charles E. Simon. The intestinal contents of each tadpole were smeared on a single slide, fixed in Schaudinn's solution and stained by the Heidenhain iron-haemotoxylin method. Nine slides were made in June 1920, ten in June 1921, and fifteen in July 1921.

3. Morphology of the trophozoite.—*G. agilis* may be described as a long slender species with the general characteristics of the genus. Its shape needs no description, being sufficiently indicated in the ventral and side views shown in Figs. 1 and 2. Measurements were made of 100 specimens taken at random from slides obtained from 17 different tadpoles. The range in length was found to be from 14.4 μ to 28.9 μ, and in width from 2.6 μ to 5.1 μ. The mean length was 22.0 μ and the mean width 4.5 μ. Measurements of different regions of 10 specimens taken at random gave the mean results presented in Fig. 3. Differences in size and shape between this species and certain other species are clearly indicated in this diagram of *G. agilis* (Fig. 3) and in similar diagrams of *G. lamblia* from man (Simon, 1922, p. 411), *G. muris* from culture rats and mice (Simon, 1922, p. 411), *G. microti* from meadow mice (Simon,

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* This is the third of a series of papers on the genus *Giardia* from the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University.

1922, p. 411), *G. duodenalis* from the rabbit (Hegner, 1922, p. 443), and *G. canis* from the dog (Hegner, 1922, p. 443).

The sucking cup of *G. agilis* is shown in ventral view in Fig. 1 and in side view in Fig. 2. It is about 4.0 μ in length and 4.9 μ in width. Anteriorly it is bounded by the anterior peristomal fibrils and posteriorly by the posterior peristomal fibrils. The posterior peristomal fibrils are quite conspicuous, contrary to the assertion of Reuling and Rodenwaldt (1921) who were unable to find them. As indicated in Fig. 1 they pass forward between the nuclei, leaving a wedge-shaped space from the center of which the ventral flagella emerge. This space or groove is similar to that described by Simon (1921) in *G. enterica* and named by him the vestibule.

The nuclei are much larger in proportion to the size of the sucking cup than those of any other species of giardia yet described. They are oval and slightly inclined toward the mid-central line at their anterior ends. A distinct nuclear membrane is present. The chromatin appears to be limited to the center of the nuclei where it forms a long pear-shaped granular mass, attached by a stem to the inner margin of the nuclear membrane at the anterior end. A rhizoplast can be seen in some specimens extending from the anterior end of the chromatin mass, at its point of attachment to the nuclear membrane, to one of the two blepharoplasts at the ends of the axostyles.

Two distinct axostyles are present, extending through the body as indicated in Figs. 1 and 2. Each is terminated at the anterior end by a blepharoplast and at the posterior end by a basal granule. The two blepharoplasts are connected by an arched commissure in the center of which is located an interblepharoplastic granule.

There are four pairs of flagella. The antero-lateral flagella originate in the blepharoplasts, cross near the anterior end of the sucking cup, pass along the anterior peristomal fibrils, and emerge on either side of the body, opposite the anterior third of the nuclei, and about 1.8 μ from the anterior end of the body. A basal granule can be seen in many specimens at the point where these flagella emerge from the body. The length of the antero-lateral flagella outside of the body is about 14 μ, or two thirds the total length of the animal.

The postero-lateral flagella also appear to originate in the blepharoplasts, from whence they pass back near the ventral surface of the body, along the inner edge of the nucleus, emerging as shown in Fig. 1 about 8.3 μ from the anterior end. Near the bases of the nuclei a distinct thickening is present where they are joined by the posterior peristomal fibrils. Outside of the body the postero-laterals are about 15 μ in length. No basal granules were observed at the points where they emerge.

The two ventral flagella arise from oval thickenings on the axostyles, a short distance back of the nuclei. They are about 15 μ long and usually parallel to each other.

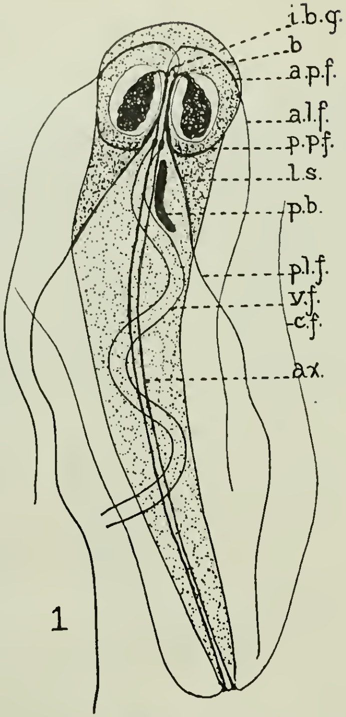
The caudal flagella extend out from basal granules at the posterior end. They appear to be merely continuations of the axostyles, and the latter probably represent their intracytoplasmic portions. They are about 17 μ long. They are shown at the sides of the body in Fig. 1, but usually stretch out posteriorly.

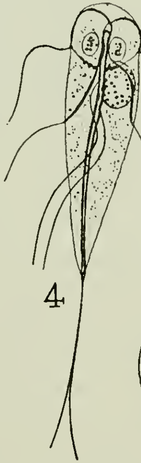
The "parabasal body" is situated usually on the left side of the body and dorsal to the axostyles, although it is often directly behind the latter. It is somewhat club-shaped, and often lies parallel to the axostyles, but usually at a slight angle to them. It is probably a single body, but in several specimens the posterior end seemed to be split. Whether this condition was due to the fixing reagent, to the presence of a double structure or to division prior to the binary fission of the animal, could not be determined. A large oval body was found by Alexeieff (1914) in certain specimens, as shown in Fig. 4, which he suggested might have arisen from the "parabasal body" ("corps médian"). No such structure appeared in any of the hundreds of specimens I examined, and it seems to me probable that this represents either an abnormality or an invading organism.

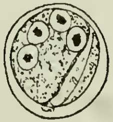
4. Cysts.—No cysts were found on my slides. Alexeieff (1914) found bodies that he considered cysts of *G. agilis* in a small frog, shortly after metamorphosis (see Fig. 5), and believes that encystment occurs at the time the tadpoles change to frogs. The cysts are then passed to the exterior in the feces and are supposedly ingested the following year by a new generation of tadpoles, thus infecting the latter. Division into two takes place within the cyst according to this author (Fig. 5).

5. Variations in length and breadth in specimens from different hosts.—While measurements were being made it was noticed that both total length and breadth were less in specimens from certain tadpoles than in those from other tadpoles. The differences noted may be indicated as follows:

With the exception of slide no. 16, the slides were made at the same time and in the same way, so that the differences in total length







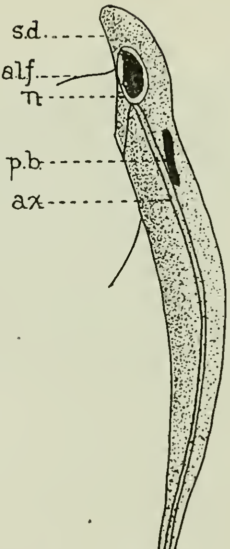


FIG. 1. Camera lucida drawing of a specimen of *Giardia agilis*, ventral view, × 4,300. *a.l.f.* = antero-lateral flagellum; *a.p.f.* = anterior peristomal fibril; *ax.* = axostyle; *b.* = blepharoplast; *c.f.* = caudal flagellum; *i.b.g.* = interblepharoplastic granule; *l.s.* = lateral shield; *p.b.* = "parabasal body"; *p.l.f.* = posterolateral flagellum; *p.p.f.* = posterior peristomal fibril; *v.f.* = ventral flagellum; *s.d.* = suction disc.

FIG. 2. Camera lucida drawing of a specimen of *Giardia agilis*, side view, × 4,300. Lettering as in Fig. 1.

FIG. 4. Ventral view of *Giardia agilis* (from Alexeieff, 1914), showing spherical body at the right just below sucking disc. × 1,500.

FIG. 5. Cyst of *Giardia agilis* (from Alexeieff, 1914). × 1,500.

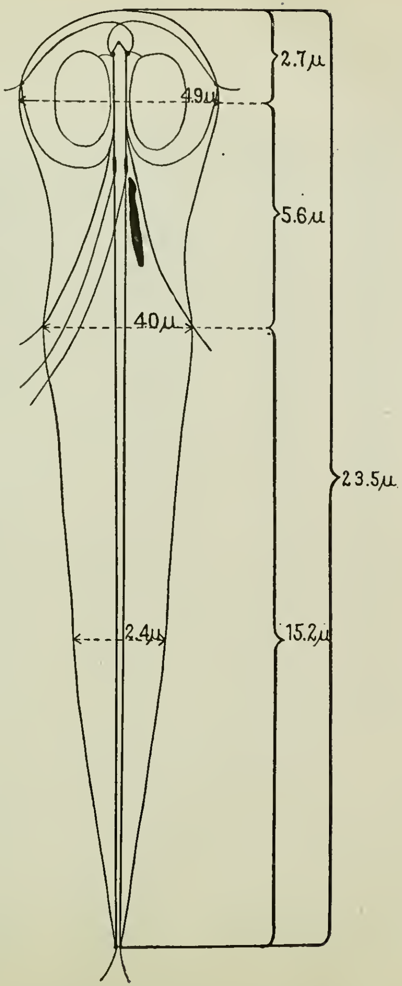


FIG. 3. Diagram of ventral view of *Giardia agilis*, × 5,400, showing average measurements of ten specimens taken at random.

are probably due to actual differences in the size of the specimens and not to modifications due to fixation. The table shows clearly that specimens on slides 11 and 16 have a mean total length about 25 per cent. greater than those on the other three slides. Width is a more difficult measurement to obtain, but it appears to be correlated with length so that the longer specimens are also broader. The greater length of these specimens is, therefore, not the result of some agent that has drawn them out antero-posteriorly at the expense of their width. The condition indicated by these measurements is similar to that pointed out by the writer (Hegner, 1921) in specimens of *Trypanosoma diemyctyli* from different individuals of the same host species, *Diemyctylus viridescens.* No definite explanation can be given to account for the differences in size observed, but heritably diverse races of *G. agilis* may exist with respect to size, or else size may be influenced by different environmental conditions in the intestines of different hosts.

No. of specimens.	No. of slide.	Range in total length.	Range in width.	Mean length.	Mean width.
15	14	15.3 μ–25.5 μ	3.4 μ–5.1 μ	19.0 μ	3.7 μ
10	10	14.4 μ–25.5 μ	4.2 μ–5.1 μ	21.0 μ	4.6 μ
10	4	18.7 μ–27.2 μ	3.8 μ–5.1 μ	21.8 μ	4.3 μ
15	11	22.9 μ–28.9 μ	4.2 μ–5.1 μ	26.1 μ	5.0 μ
10	16	25.5 μ–28.9 μ	5.1 μ–5.1 μ	26.4 μ	5.1 μ

6. *Giardia agilis* AND *Giardia alata.*—Kunstler and Gineste in 1907 described a second species of giardia from tadpoles which they called *G. alata.* They had difficulty in staining specimens, both of this form and of *G. agilis,* and I attribute to this difficulty their conclusion that they were dealing with two species. That *G. agilis* and *G. alata* are the same species has also been expressed by other writers. If there is only one species in the tadpole the laws of nomenclature give *G. agilis* priority.

7. SYSTEMATIC RELATIONSHIP OF *Giardia agilis* FROM THE TADPOLE AND *Giardia lamblia* FROM MAN.—Reuling and Rodenwaldt (1921) have recently studied *G. agilis* from tadpoles collected at Heidelberg, and have compared its form, structure, and locomotion with the species occurring in man. They note the following differences in the two species: (1) in the shape of the body, (2) in the form of the sucking disc,

that of *G. agilis* being without the posterior peristomal fibrils, (3) in the blepharoplasts, there being two in *G. agilis* and four in the human form, (4) in the position and form of the "parabasal bodies," that of the human form being double and situated behind and at an angle with the axostyle, whereas that of *G. agilis* is single and parallel to the axostyle, (5) in the shape of the posterior end, and (6) in the method of locomotion. The conclusion reached by these investigators is that these differences are important enough to separate the two species and to place them in two subgenera, namely "Subgenus *Giardia s. str.*, schlanke Formen mit kopfartigem Vorderende, querovalem maulartig vertieftem Peristom und kolbenartigen längsgestellten Parabasale mit charakteristischen Krümmungsbewegungen, und das Subgenus *Lamblia,* gestauchte, birnförmige Flagellaten, mit nierenförmigem Peristom, Vorderkörper flach schalenförmig, Parabasale schräg quergestellt und spangenförmig, Bewegung schaukeld" (Reuling and Rodenwaldt, 1921, p. 345).

My findings differ in several respects from those reported by these investigators and I cannot agree with their conclusions. I can find no bridge extending over the vestibule such as they figure. The sucking cup in my material does not differ in *G. agilis* from that in other species of giardia, but is a sucking disc bounded by anterior and posterior peristomal fibrils, as indicated in my figures. As in certain other species of giardia, I find an interblepharoplastic commissure, in the center of which a distinct granule is located, and basal granules where the antero-lateral and caudal flagella emerge from the body. The location of the "parabasal body" in *G. agilis* is parallel to the axostyle and single, instead of double, as in some other species of giardia, but in my material several specimens were found in which it appeared to be split at the end. The real differences, therefore, between the species from the tadpole and that from man are the shape of the body, and the position, shape and single condition of the parabasal of *G. agilis*. These, in the opinion of the writer, are sufficient to separate the two forms as distinct species, but not as genera or subgenera. The following measurements indicate the differences in size of the two species.

8. SUMMARY.—(1) *Giardia agilis* from the tadpole is similar to *Giardia lamblia* from man in structural characteristics, and the differences in size and shape between the two species are not sufficient to place them in separate genera or subgenera.

Measurements of Giardia lamblia from man (from Simon, 1921) and Giardia agilis from the tadpole.

Species.	No. of specimens.	Length of body.			Breadth of body. Mean.
		Lowest.	Highest.	Mean.	
G. lamblia......	342	9.25	20.25	13.70	7.5
G. agilis.......	100	14.4	28.9	22.0	4.5

(2) Specimens of *Giardia agilis* from different tadpoles sometimes exhibit uniform differences in size. These differences may be due to the presence of heritably diverse races, as regards size, or to changes brought about by the different environmental conditions within the intestines of their tadpole hosts.

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A COMPARATIVE STUDY OF THE GIARDIAS LIVING IN MAN, RABBIT, AND DOG.*

By ROBERT W. HEGNER, Ph.D.

(Received for publication, April 25, 1922.)

The presence of giardias in a number of the lower animals, dogs, cats, rabbits, rats, mice, etc., was considered until recently to indicate the probability of cross infection and to account largely for the spread of these flagellates among human beings.

The following comparative study was made in order to determine more accurately than has been done heretofore the structural characteristics and size and shape of the giardias found in the rabbit and dog. These data are contrasted with those obtained by Simon (1921) for the giardia from man, and the conclusion is reached that each animal is infected with a separate species. The species in man is *Giardia lamblia* (Stiles, 1915) [Kofoid and Christiansen, 1915]; that in the rabbit is *Giardia duodenalis* Davaine, 1875; and that in the dog *Giardia canis* sp. nov.

Material and methods.—(1) *Giardia duodenalis.* Laboratory rabbits supplied an abundance of trophozoites of this species. These were fixed in Schaudinn's fluid and stained by Heidenhain's iron-haemotoxylin method. (2) *Giardia canis.* Trophozoites and cysts of this species were obtained from four dogs, about three months old, purchased from an animal store in Baltimore. All four were suffering from diarrhoea. One of them died at the time of purchase; another died soon after; but the other two recovered in a few weeks. Examinations of the intestinal contents of the dogs that died revealed infection also with *Amoebae, Trichomonas, Ascaris,* tapeworm, and hookworm.

Giardia duodenalis Davaine, 1875.

Morphology of trophozoite.—*Giardia duodenalis* resembles in general shape the species that have been recorded from man and other warm-blooded animals. In details, however, it differs in several dis-

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* This is the fourth of a series of papers on the genus *Giardia* from the Department of Medical Zoology of the School of Hygiene and Public Health, Johns Hopkins University.

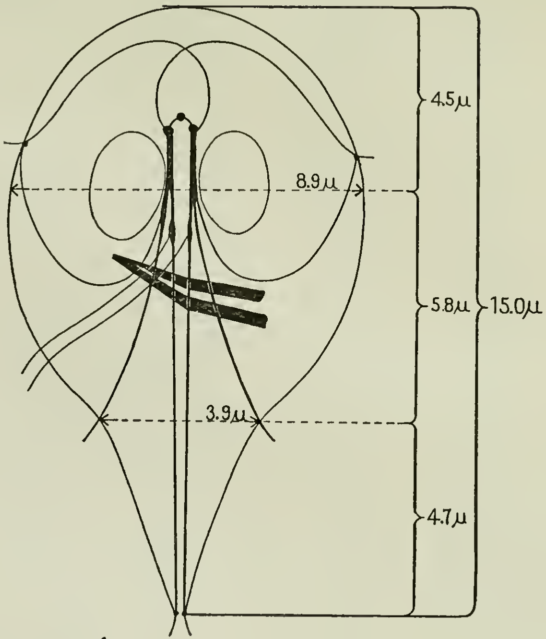

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FIG. 1. Diagram of ventral view of *Giardia duodenalis*, × 5,400, showing average measurements of ten specimens taken at random.

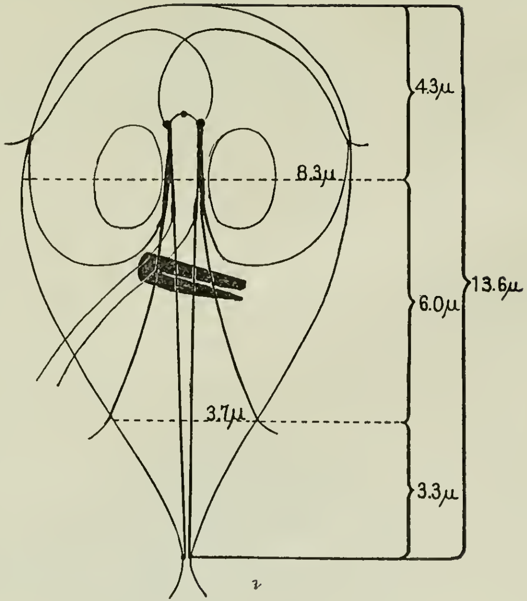

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FIG. 2. Diagram of ventral view of *Giardia canis*, × 5,400, showing average measurements of ten specimens taken at random.

tinguishable and constant characteristics. These may best be brought out by comparing the outline of this species, shown in Fig. 1, with that of the species found in the dog, Fig. 2. The body of *Giardia duodenalis* is narrower in the region anterior to the nuclei, but conspicuously broader across the center of the lateral shields. The distances from the anterior end to the center of the nuclei, and from the center of the nuclei to the point of emergence of the postero-lateral flagella are almost the same in both species, but the distance from the latter point to the posterior end—the region that we may speak of as the tail—is much greater in *Giardia duodenalis* than in *Giardia canis.*

Measurements were made of 50 specimens, taken at random from a number of slides, prepared from material from a single rabbit. The range in length was found to be from 12.7 μ to 18.7 μ and in width from 7.7 μ to 11.0 μ. The mean length of these specimens was 15.8 μ and mean width 9.1 μ. Further measurements of ten specimens taken at random gave the results indicated in Fig. 1, i.e., total length 15.0 μ; from anterior end to center of nuclei, 4.5 μ; from center of nuclei to end of lateral shields, 5.8 μ; from end of lateral shields to posterior end of body, 4.7 μ; width at center of nuclei, 8.9 μ; and width at ends of lateral shields, 3.9 μ.

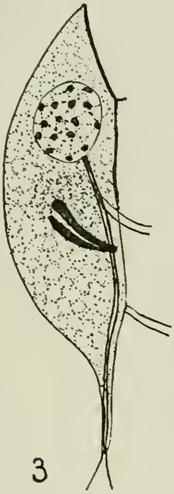
When compared in size and shape with the human giardia, *Giardia duodenalis* is seen to be on the average both broader and longer as shown in the measurements in Table I.

TABLE I.

Measurements of the trophozoites of Giardia duodenalis from the rabbit, and Giardia canis from the dog, compared with those of Giardia lamblia (Simon, 1921) from man. Length and breadth are given in microns.

Species.	No. of specimens.	Length of body.			Breadth of body.			Ratio of body length to breadth.
		Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.	
G. lamblia..........	342	9.25	20.25	13.70	5.0	10.25	7.5	1.84
G. duodenalis.......	50	12.7	18.7	15.8	7.7	11.0	9.1	1.74
G. canis............	50	11.9	17.0	13.8	7.6	10.2	8.5	1.62

A side view of *Giardia duodenalis* is shown in Fig. 3. The shape when seen from the side differs considerably according to the position of the tail and of the sucking cup, but the specimens of *Giardia duodenalis* examined seemed to be thicker near the anterior end and thinner in the region of the "parabasal bodies" than those of *Giardia canis* (compare Figs. 3 and 11).



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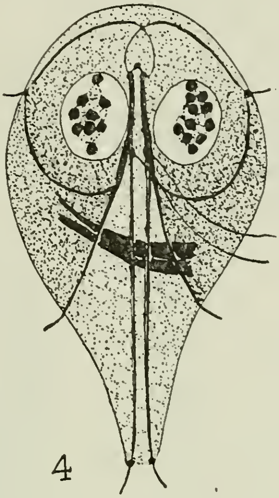

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FIG. 3. Side view of *G. duodenalis*. The nucleus is large and almost spherical, with chromatin masses rather evenly distributed. Compare with side view of *G. canis*, Fig. 11, page 450.

FIG. 4. Ventral view of *G. duodenalis*. Two blepharoplasts are present. The chromatin within the nucleus has broken up into a number of masses of approximately equal size. The axostyles show evidence of splitting. Camera drawings, × 4,300. Only basal parts of the flagella are shown. See Fig. 5, page 446, for key to structures shown.

It does not seem necessary to describe in detail the various structures of this organism, hence only a few will be selected for emphasis. Two axostyles are invariably present. Each of these seems to be terminated at the anterior end by a single blepharoplast in specimens that are not in some stage of division (Fig. 1). In other specimens in which the chromatin within the nucleus has begun to spread out from the central karyosome four distinct blepharoplasts are often visible (Fig. 5). The ends of the antero-lateral flagella are attached to the outer two; the inner two are connected by an interblepharoplastic commissure, in the center of which is situated an interblepharoplastic granule; and a single fibril extends posteriorly from each of the four blepharoplasts, its identity becoming lost in the deeply staining material between the nuclei. Basal granules are often visible where the antero-lateral and caudal flagella emerge from the body, and sometimes what appears to be a granule is visible where the antero-lateral flagella cross within the body. There are two "parabasal bodies" posterior to the axostyles, with their right ends usually united and pointed and the left ends usually more or less widely separated and blunt. Just dorsal to the axostyles these "parabasal bodies" are often bent, which gives them a very characteristic appearance. They differ also from similar structures in other giardias in their great length, some of those measured being over 5 μ long.

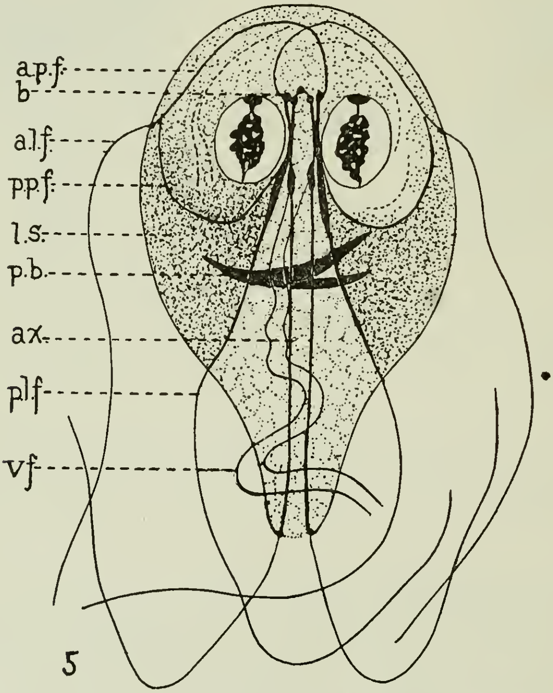


FIG. 5. Ventral view of *G. duodenalis* showing length of all of the flagella. Four blepharoplasts are present; the chromatin within the nuclei is breaking up into small masses; and lines that may represent duplicated flagella or peristomal fibrils are present in the region of the sucking cup. Camera lucida drawing, × 4,300. *a.l.f.* = antero-lateral flagellum; *a.p.f.* = anterior peristomal fibril; *ax.* = axostyle; *b.* = blepharoplast; *l.s.* = lateral shield; *p.b.* = "parabasal body"; *p.l.f.* = postero-lateral flagellum; *p.p.f.* = posterior peristomal fibril; *v.f.* = ventral flagella.

The length of the four pairs of flagella is indicated in Fig. 5. Three stages in the arrangement of the chromatin, prior to nuclear division, are shown in Figs. 3, 4, and 5.

Davaine (1875) was the first to describe a giardia from the rabbit, naming it *Hexamita duodenalis.* Grassi and Schewiakoff (1888) also report a giardia from rabbits, but considered it the same species as in man.

The first extended study of the giardia of the rabbit, reported in the literature, is that of Metzner (1901) who considered it the same species as that occurring in man and applied to it the name *Megastoma entericum* Grassi. Metzner's technique did not include the iron-haemotoxylin method and failed to reveal certain details in structure. The general proportions of the body, however, were correctly ascertained, thickenings were observed where the ventral flagella arise, and granules were noted at the bases of the caudal flagella.

Bensen (1908) was the first to recognize the giardia of the rabbit as a distinct species to which he gave the name *Lamblia cuniculi.* He does not give measurements but states that it is somewhat smaller and broader than the form occurring in man. Bensen's figures of this species agree fairly well with my own, differing principally in the absence of the interblepharoplastic granule and the oblong thickenings where the ventral flagella arise, and in the smaller size of the "parabasal bodies." The "tail" in his figure is shorter than I have found it to be in my specimens.

The specific name *cuniculi,* applied to this species by Bensen, is not valid, since the name *duodenalis* was given to the giardia in the rabbit by Davaine in 1875. The term *cuniculi* is, however, erroneously retained by certain investigators (Kofoid and Swezey, 1922, pp. 222 and 223).

Fonseca (1915) reports *Giardia* (*cuniculi*) *duodenalis* from the intestine of two rodents from Brazil, *Coendu villosus* and *Oryctolagus cuniculus.* The body according to Fonseca is from 10–20 μ in length and from 6–12 μ in width. These measurements agree fairly well with my own, although the range is greater, but no real comparison can be made since Fonseca does not state the number measured nor give the mean length and breadth. The figure given by Fonseca (Fonseca, 1915, Fig. 23) presents a shape that does not resemble the form that I have studied nor that figured by Metzner (1901) and Bensen (1908), the anterior portion of the sucking disc being too broad and the diameter across the middle region of the lateral shields being too narrow.

Fonseca omits the basal granules where the antero-lateral and caudal flagella leave the body, but includes a granule where the antero-laterals cross within the body. He also fails to show the thickening of the postero-lateral flagella just opposite the bases of the nuclei, which are clearly indicated by Bensen and are quite conspicuous in my own material. It seems probable to the writer that Fonseca was not dealing with *Giardia duodenalis,* but with another species.

The separation of the giardias into three species by Bensen (1908) has met with the approval of most investigators. Noc (1908), however, thinks that he has found among specimens from man all of the characteristics used by Bensen in separating species. Simon (1921), however, has provided an adequate description of *G. lamblia* from man, and more recently (Simon, 1922) has proved by observation and experiment that *G. muris* is a distinct species. The third of Bensen's species, *G. duodenalis,* is shown by my own studies to be different from the other two, thus confirming completely Bensen's results.

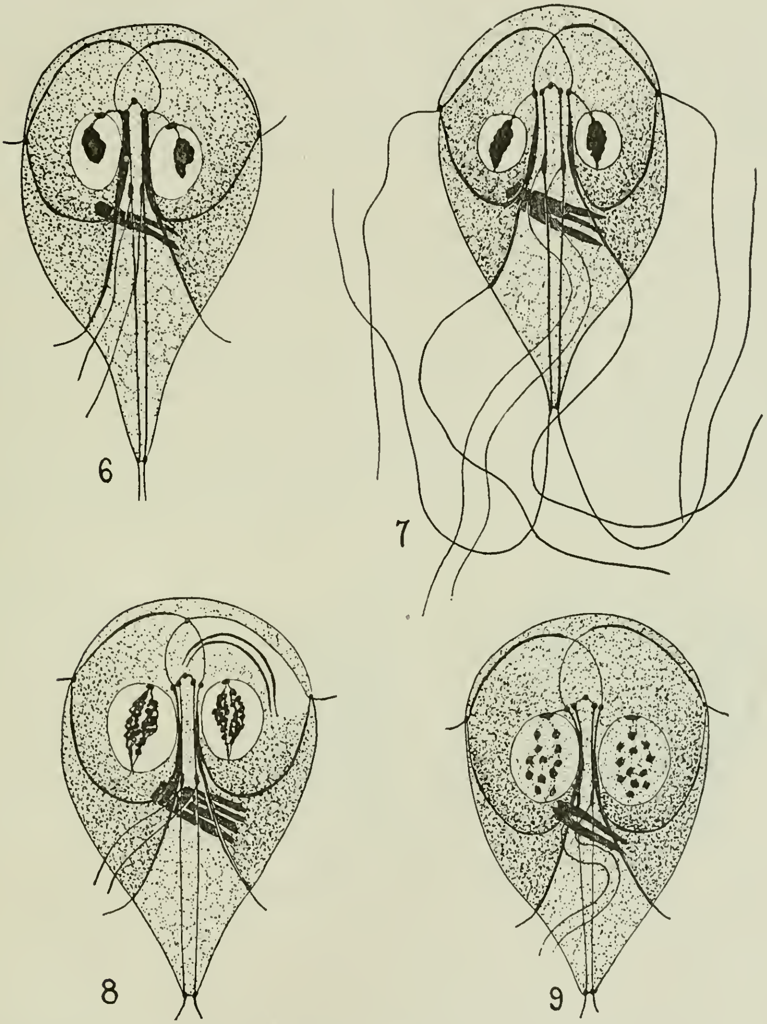
GIARDIA CANIS SP. NOV.

Giardias have not been reported very often from dogs. Grassi and Schewiakoff (1888) list the dog as one of the animals in which they were found, and Janowski (1897) mentions finding giardias in dogs and cats that measured from 15 μ to 20 μ in length.

The giardia of the dog differs both in shape and size from the species that have been described from other animals.

Size has been found by many investigators of the protozoa to be a very constant heritable characteristic of these organisms. Slight differences in shape are also heritable and constant (Hegner, 1919a, 1919b). These differences, therefore, may be considered of specific value, and I therefore propose the specific name *canis* for this form. The type and cotypes are deposited in the collections of the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University. One of the striking features of *G. canis,* when compared with *G. duodenalis* and *G. lamblia,* is its broad anterior end (Fig. 2). It is about the same length as *G. lamblia,* having a mean length of 13.8 μ, as compared with 13.7 μ for *G. lamblia* (Simon, 1921), but is considerably broader, with a mean of 8.5 μ, as compared with 7.5 μ in *G. lamblia.*

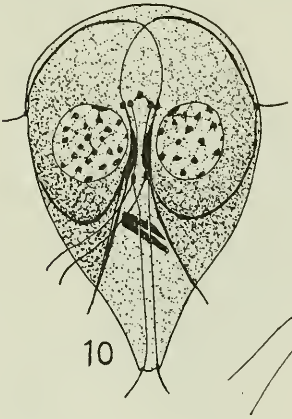
Morphology of the trophozoite.—The range in total length of 50 specimens of *G. canis,* taken at random, was 11.9 μ to 17.0 μ, and in width 7.6 μ to 10.2 μ. The mean total length of these 50 specimens was 13.8 μ and mean width 8.5 μ. The mean measurements of ten specimens are given in Fig. 2.



Figs. 6–9. Ventral views of *G. canis.* Camera lucida drawings, × 4,300. Fig. 6 shows condition before division begins, with two blepharoplasts and two "parabasal bodies." Fig. 7 shows the flagella *in toto;* the chromatin within the nuclei has begun to spread out; and the parabasal bodies have divided. Fig. 8 shows a later nuclear stage, with increased size of nucleus and splitting up of chromatin into small masses; further division of "parabasal bodies"; four blepharoplasts; and duplication of fibrils in region of sucking disc. Fig. 9 shows later stage in growth of nucleus and distribution of chromatin masses.

The various structures of *G. canis* differ but slightly from those of other species that have been described. When division is not in progress (Fig. 6) the chromatin is condensed into an irregular central mass in each nucleus and attached to a disc-shaped granule on the inside of the nuclear membrane at the anterior end. There is a single blepharoplast at the anterior end of each axostyle, and an interblepharoplastic commissure with a centrally located granule is present. The two "parabasal bodies" lie obliquely dorsal to the axostyles, with their blunt right ends united and their more pointed left ends separated slightly.

Early division stages were abundant in my material. In these, four distinct blepharoplasts were visible. No stages were found in which the axostyles were divided, but in several cases (Figs. 8 and 10) the intracytoplasmic portions of the postero-lateral flagella were divided, and in many cases one or two curved fibrils were visible on the left side near the anterior end of the body. The presence of a very faintly staining streak along the antero-lateral flagellum on this side (Fig. 8) suggested that these fibrils might represent the left peristomal fibril that had separated from the antero-lateral flagellum and divided into two.



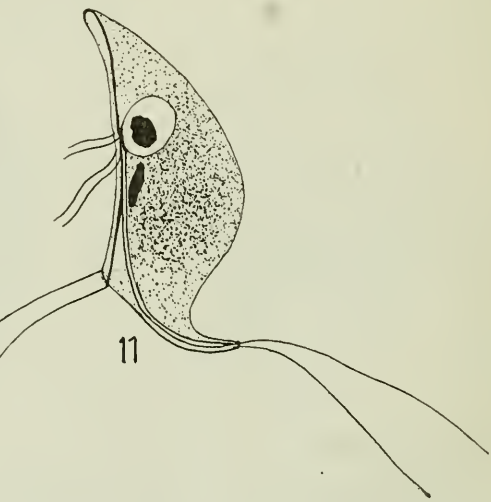


Fig. 10. Ventral view of *G. canis* showing four blepharoplasts each at the end of a fine fibril; the intracytoplasmic portion of the right postero-lateral flagellum split; large spherical nuclei with evenly distributed chromatin masses. Camera lucida drawing, × 4,300.

Fig. 11. Side view of *G. canis*. Camera lucida drawing, × 4,300. Compare with similar view of *G. duodenalis* shown in Fig. 3.

Various nuclear conditions were observed. These are probably genetically connected as follows: The "resting" condition is illustrated in Fig. 6. The central karyosome lengthens until it extends across the long diameter of the nucleus (Fig. 7). The chromatin granules in each chromatic mass then separate from one another and appear to form two irregular strands (Fig. 8); further separation of the chromatin granules continues (Fig. 9), until they become approximately equally distributed throughout the nuclei (Fig. 10). During this process the nuclei increase in size and become almost spherical in shape (compare Figs. 7 and 10).

In some specimens the "parabasal bodies" were evidently undergoing or had undergone division since three or four were visible (Figs. 7 and 8).

A lateral view of *G. canis* is shown in Fig. 11. It differs considerably from the similar view of *G. duodenalis*, illustrated in Fig. 3.

Cysts.—Cysts of *G. canis* were abundant. They are oval in shape and all of those I observed contained four nuclei. These were situated in most specimens at one end (Figs. 12 and 13), but many cysts were present with two nuclei at each end (Fig. 14). The 50 cysts measured ranged in length (exclusive of the cyst wall) from 9.4 μ to 12.7 μ and in width from 6.8 μ to 8.9 μ. The mean length was 11.1 μ and mean width 8.2 μ.

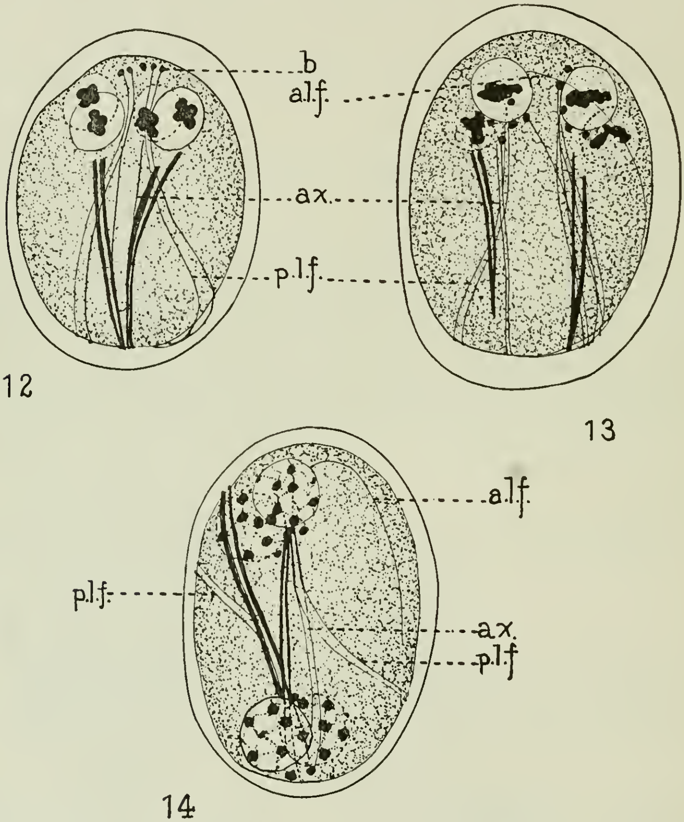
The measurements of these cysts are compared with those furnished by Simon (1921) for *G. lamblia* in Table II.

TABLE II.

Measurements of the cysts of G. canis from the dog, compared with those of G. lamblia from man. Length and breadth are given in microns.

Species.	No. of specimens.	Length.			Breadth.			Ratio of length to breadth.
		Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.	
G. lamblia..........	250	8.0	14.0	10.7	6.0	10.0	7.47	1.37
G. canis............	50	9.4	12.7	11.1	6.8	8.9	8.2	1.35

Because of the remarkable clearness of the stained specimens and the absence of certain fibrils that are characteristic of the cysts of some of the other species of giardia which have been described it was a comparatively simple matter to determine the number and location of the fibrils. Nevertheless I was unable to convince myself as to the identity of the various fibrils, and hence suggest the following as their probable but not certain origin. In specimens with four nuclei at one end (Figs. 12 and 13) there are visible two pairs of divergent fibrils that I believe to be divided postero-lateral flagella. Sometimes two and sometimes two pairs of parallel fibrils can be seen; these appear to be axostyles. Two pairs of heavier fibrils separated at the end where the nuclei are located, but united at the opposite end,



FIGS. 12–14. Cysts of *G. canis.* Camera lucida drawings, × 4,300. In Figs. 12 and 13 there are four nuclei at one end; in Fig. 14, two at each end. The identity of the various fibrils cannot be definitely stated but are tenatively labelled as follows: *a.l.f.* = antero-lateral flagellum; *ax.* = axostyles; *b.* = blepharoplast; *p.l.f.* = postero-lateral flagella.

resemble similar bodies that occur in the cysts of the human species and that have been interpreted by Simon (1921) as ridges of the lateral shields. This may be the correct identification, but it seems more probable to me that they are peristomal fibrils such as are found in the early division stages of the trophozoite (Fig. 8). Several other fine fibrils that were observed in the cysts probably represent antero-lateral flagella. Granules that are probably blepharoplastic in nature are present near the nuclei, but their relationship to the various fibrils could not be determined with certainty.

In cysts that possess two nuclei at each end similar sets of fibrils occur, but these have evidently shifted with the nuclei as shown in Fig. 14.

The impression was gained from my material that division is already in progress when *G. canis* encysts and is continued within the cyst wall. No trophozoites with more than two nuclei were encountered and no cysts with less than four were observed. The nuclear conditions such as are shown in Figs. 12, 13 and 14 suggest the fusion of chromosomes (Figs. 12 and 13) and subsequent reorganization (Fig. 14). In some cases definite tetrads were noted (Fig. 12).

SUMMARY.

1. *Giardia duodenalis.*—This species is characterized by a narrowing of the anterior region, as seen in front view, and a broadening across the center of the lateral shields, by a comparatively long "tail" and by two long bent "parabasal bodies" (see Fig. 1). It is both broader and longer than the species, *G. lamblia,* from man, but the difference in the ratio of body length to body breadth shows that the latter is more slender than *G. duodenalis.* The data recorded confirm the opinions expressed by several previous investigators that the rabbit is parasitized by a species distinct from that living in man.

2. *Giardia canis.*—This form which has been recorded from the dog by several investigators represents a distinct species to which the specific name *canis* is given. The trophozoite differs from that of *G. lamblia* from man and *G. duodenalis* from the rabbit principally in size, shape, and the form of the "parabasal bodies." The anterior end of the trophozoite in front view is broader than in the other two species; the diameter across the center of the lateral shields is less than in *G. duodenalis;* the two "parabasal bodies" are longer than the parabasals of *G. lamblia,* but shorter than those of *G. duodenalis;* and the "tail" is shorter than in *G. duodenalis,* but about the same

relative length as in *G. lamblia*. The cysts of *G. canis* are of the same shape as those of *G. lamblia*, but slightly larger both in length and breadth.

A good idea of the differences in size, shape, and structure of the various species described in this paper may be obtained by referring to the diagram of *G. lamblia* (Simon, 1922, p. 411), of *G. agilis* (Hegner, 1922, p. 438), of *G. duodenalis* and *G. canis* (p. 443) and to the measurements of *G. agilis* (Hegner, 1922, p. 441), of *G. duodenalis* (p. 444), and of *G. canis* (pp. 444 and 451).

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PROCEEDINGS OF THE SOCIETY OF HYGIENE OF THE SCHOOL OF HYGIENE AND PUBLIC HEALTH OF THE JOHNS HOPKINS UNIVERSITY, BALTIMORE, MARYLAND.

Regular Meeting of Monday, April 10, 1922.

Carriers of Virulent Diphtheria Bacilli in Baltimore. By J. A. Doull. From the Department of Epidemiology and Public Health Administration, School of Hygiene and Public Health, Johns Hopkins University. in cooperation with the City Department of Health of Baltimore.

Most studies on the distribution of the diphtheria bacillus fail for one of the following reasons: First, a fair sample of the population to be studied is not taken; the sample is not selected merely by simple sampling, and it is usually too small. Second, there are many differences in diagnostic criteria, and usually only a small proportion if any of the organisms are tested on animals. Third, the age and sex constitution of the population studied and of the carriers found is not recorded. Fourth, the prevalence of the disease in the area is not studied, i.e., epidemic, seasonal, and local prevalence.

The work undertaken here consists of: (1) An epidemiological study of cases as reported, including an examination in 1921 by the Department of Immunology of this school of cultures taken from family contacts. (2) A survey of groups of school children to determine the incidence in the school population of carriers of virulent organisms with regard to season, color, sex, age, the condition of the throat, history of the disease or family contact within 12 months. In the schools each sixth child was taken, combined cultures being made from separate nose and throat swabs. These cultures also were examined by the Department of Immunology, suspicious organisms being obtained in pure culture and virulence being determined by the intracutaneous test.

1. *With regard to family contacts:* The material is from a series of 103 primary cases occurring chronologically in the spring of 1921. Five hundred and thirteen persons were considered contacts

and 81 per cent. were cultured. In the age group under 5 there is a ''family attack rate'' of 19.6 per cent., in the group 5–9, a rate of 38.7 per cent., and then there is a rapid decline in the number of virulent carriers as age increases. With regard to sex below the age of 15 there is no significant difference, while over 15 there is a rate of 9.5 per cent. among females to 3.2 per cent. among males. The annual reports of the City Health Department of Providence show that the secondary attack rate (cases) exhibits much the same peculiarity. The Providence figures are for the years 1896-1913. Under 5 the rate is 19.7 per cent., 5–9 it is 18.8 per cent., under 15 there is no material difference between the sexes, while females over 15 show a rate of 4.6 per cent. compared to 1.9 per cent. among the males.

The age and sex groups which tend to become cases also tend to become carriers. This is very important in the control of the disease.

2. *Concerning carriers in the school population:* Of 635 boys found at the swimming pool at Clifton Park and cultured (throats) by members of the staff of the Department of Immunology, only one was found to be a virulent carrier. This was in July 1921. In November and December over a period of six weeks, 2,216 school children were cultured (combined). Seventy-three were found to harbor virulent organisms or 3.1 per cent. There was nothing significant about any age, sex, or color group. In February–March over a period of 7 weeks, 2,350 children were cultured. The sample is strictly comparable to the other in every way. The number of carriers found was 34 or 1.45 per cent. If the census figure for 1920 be taken, there are in the six wards 26,754 children of the ages 7–15, inclusive. Including only carriers of these ages at the November–December rate, there would be at one time 936 carriers and at the February–March rate, only 401. In the seven weeks from the beginning of the first survey there were 36 clinical cases of these ages and in the same period from the beginning of the second there were only 14 cases. There is a ratio of carriers to cases of 26 to 1 for the first period and of 28 to 1 for the second. It is hoped to carry out another school survey in May.

An interesting fact is that while in the families of 343 clinical cases which have been investigated by us there were 26 secondary clinical cases, there has been only one clinical case reported from the families of the 104 virulent carriers discovered since November.

The Use of the Original Diagnostic Culture for the Determination of the Virulence of Diphtheria Bacilli. By LEON C. HAVENS, M.D. From the Department of Immunology, School of Hygiene and Public Health, Johns Hopkins University.

The methods in use for determining the virulence of diphtheria cultures depend on the isolation in pure culture of the organism, and thus represent, usually, only a single colony rather than the whole culture which is present in the individual's throat.

The original diagnostic culture has been used to determine virulence in a series of 509 cultures. Pure cultures were used to check the results obtained with the whole cultures, using the intracutaneous method of Zingher and Soletsky.* The cultures were from patients, contacts, and healthy individuals not associated with cases, and were collected throughout different seasons of the year.

After the microscopic examination the culture was suspended in salt solution, the amount varying, depending on the relative numbers of diphtheria bacilli in proportion to other organisms in the culture. Six cultures can be tested on large guinea pigs, four on smaller ones. Controls injected previously with antitoxin are essential. 0.1 c.c. of the suspension is injected intracutaneously into the test animal and a like amount in a corresponding position into the control. Readings are made at intervals of 24, 48, and 72 hours, the lesions on the test and control animals being compared. The only dependable criterion of a positive test is necrosis of the superficial layers of the skin in the test animal, this lesion being absent in the control. The size of the lesion is not important, the size of the necrotic area being due largely to the relative numbers of diphtheria bacilli injected. Frequently the necrosis will develop in 24 hours and usually by 48 hours, thus resulting in a saving of time and labor spent in isolating pure cultures.

Of the 509 cultures tested by this method, 341 were positive by microscopic examination and 168 were negative. None of the microscopic negatives gave a positive virulence test, while 305 of the microscopically positive cultures gave a positive test and 36 a negative test. Pure cultures were isolated from all but 46 or 295 of the microscopically positive cultures and were tested for virulence by the intracutaneous method. 263 of the 295 pure cultures were virulent and 32 gave negative tests. 275 (93 per cent.) of the cultures gave the same results by both methods. There were 20 discrepancies. Eight were positive with the whole culture and negative with the pure culture; 12 (4 per cent.) were negative with the whole culture method and positive in the pure culture, probably due to the small numbers of diphtheria bacilli present in the original culture. The 8 cultures that were positive by the whole culture method and negative in pure culture cannot be counted against the method since it is possible that the cultures contained both virulent and avirulent organisms, the pure culture being obtained from colonies of the latter. A series of cultures were plated and a large number of colonies was tested for virulence. Five of 18 original cultures were thus found to contain both virulent and avirulent organisms.

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* Zingher, A., and Soletsky, D., 1915, J. Inf. Dis., XVII, 455.

Pure cultures could not be isolated from 46 (13 per cent.) of the cultures. These would not have been tested had the pure culture method been used. In other words, the whole cultures method permits the testing of all cultures for virulence.

In order to determine what influence, if any, the common contaminants have on the outcome of the test, a pure culture was selected and the smallest number of organisms which would produce a positive result was determined by actual count. This was found to be 400,000, 300,000 and 200,000 failing to cause lesions. When mixed with varying proportions of staphylococci or streptococci, either in equal numbers or up to 1 diphtheria bacillus to 100 of the other organisms, a positive test resulted with only 200,000 diphtheria bacilli. This apparent enhancement of virulence is probably due to the fact that the destruction of the diphtheria bacilli is retarded, giving them a longer time in which to produce toxin. A less probable explanation is that the cocci render the tissues more susceptible to the necrotizing action of the toxin.

Observations on Single Cell Cultures of B. diphtheriae. By H. M. Powell. From the Department of Immunology, School of Hygiene and Public Health, Johns Hopkins University.

A biological study of the diphtheria bacillus which has been carried on for somewhat more than a year through observations made primarily upon 38 "colony" cultures of this organism, together with 304 single cell cultures derived with the aid of the Barber technique from the colony cultures, has indicated that both single cell and colony cultures produce morphological types which, although they fluctuate somewhat, present in most, if not all cases a fairly definite morphological picture from generation to generation. Regular observations have been made on all cultures in regard to morphology, cultural reactions, and virulence. All cultures, both colony and single cell, have continuously produced acid in dextrose, maltose, and dextrin serum water media, while no change resulted at any time in lactose or saccharose serum water. Also all have constantly given the same kind of virulence test, i.e., those which were originally virulent remained so and vice versa. No culture has been "split" through study of its morphology, cultural reactions, virulence, or agglutination or the study of these properties of the single cell cultures derived from any original culture. All single cell cultures derived from an original colony or single cell culture agglutinate to the same titer as the original culture does. Part of the cultures used in the study as well as several additional cultures have thus far been classified by agglutination reactions, and no cross agglutination between virulent and avirulent organisms has been encountered. Observations are being continued on these different characteristics of the organism.

Observations on Two Dissimilar Diphtheria Toxins. By Ida W. Pritchett. From the Department of Immunology, School of Hygiene and Public Health, Johns Hopkins University.

Two diphtheria filtrates have been studied. The first, designated as Toxin 1, was made October 5, 1920, with a transplant of *B. diphtheriae* Park 8 and showed, during the first six months of its use, the following measurements:

M. L. D.	.004	c.c.
L–o dose	.19	c.c.
L–plus dose	.33	c.c.
L–plus minus L–o	35	m.l.d.

The second, designated as Toxin 2, was made February 8, 1922, with a second transplant of *B. diphtheriae* Park 8 and showed, during the first two months of its use, the following measurements:

M. L. D.	.0035	c.c.
L–o dose	.11	c.c.
L–plus dose	.15	c.c.
L–plus minus L–o	about 11	m.l.d.

The measurements indicate that the two toxins differed materially not only in potency, but also, and to a more marked degree, in their so-called toxon fraction, Toxin 1 showing a difference of 35 m.l.d. between the L–o and the L–plus doses and Toxin 2 a difference of only about 11 m.l.d.

Certain striking differences were observed in the action of the two toxins. It was found that Toxin 1 produced a slight skin lesion in 250 gram guinea pigs when injected subcutaneously in quantities as small as 1/10,000 to 1/15,000 of a c.c., while 1/1,000 c.c. produced quite a massive local oedema. It was also found that this toxin, when mixed with one unit of antitoxin, in quantities all the way from the L–o to L–plus dose, was able to produce in the kidneys of guinea pigs swelling and proliferation of Bowman's capsule even in mixtures corresponding to, or very slightly exceeding, the L–o dose, while the pure toxin produced similar swelling and proliferation of Bowman's capsule in quantities as small as 1/1,000 c.c., and to a slight degree even at 1/5,000 c.c., but failed to do so in higher dilutions even though it was capable of causing a local skin lesion in such dilutions. Toxin 2 contrasted strongly with Toxin 1 in that, though somewhat more potent in respect to its killing power, it failed to cause a local skin lesion, when injected subcutaneously into 250 gram guinea pigs, in quantities as small as 1/1,000 c.c., but produced a marked lesion at 1/600 c.c., at which point also there was swelling and proliferation of Bowman's capsule. Like Toxin 1, it was able, when mixed with one unit of antitoxin, to cause swelling and proliferation of Bowman's capsule in neutral, or nearly neutral, mixtures of toxin and antitoxin.

Some intracutaneous tests were run in guinea pigs with these two toxins to see how their toxicity compared by this method. The results bore out absolutely the above findings. It was found that Toxin 1, at this time some 18 months old and showing an m.l.d. of about .0055 c.c., produced necrosis when injected intracutaneously in a quantity corresponding to about 1/300 c.c., while Toxin 2, with an m.l.d. of .0035 c.c., failed to produce necrosis in quantities corresponding to dilutions above 1/250 c.c. It is evident that, at least with these two toxins, an accurate estimate of potency would have been impossible by the intracutaneous method.

It is generally assumed that toxin-antitoxin mixtures are gradually dissociated *in vivo*, the toxin thus freed combining with susceptible body cells and thus giving rise to the active production of antitoxin.

While the experiments undertaken in the course of this work have failed to prove that such a dissociation does not take place, it is felt that the fact that swelling and proliferation of Bowman's capsule occur in guinea pigs injected with neutral, or nearly neutral, mixtures of toxin and antitoxin, while at the same time it fails to occur in guinea pigs injected with pure toxin until after the establishment of a distinct local lesion in the skin, suggests that there may be present in the toxic filtrate some specific harmful substance other than the one which causes acutely fatal intoxications, the substance being carried to the cells of Bowman's capsule in the excreted urine. This hypothetical toxic fraction appears, in the present instance, to be associated with a high toxon content, and to be relatively low in the toxon-deficient filtrate.

Experiments are now being conducted to determine whether Toxin 2, when injected subcutaneously into guinea pigs in combination with one unit of antitoxin (L–o to L–plus mixtures), will cause late paralyses in these animals with the same ease as toxins richer in toxon. If it fails to do so, it is thought that this would indicate that the paralysis-producing substance (toxon?) and the substance causing lesions of Bowman's capsule in neutral, or nearly neutral, mixtures of toxin and antitoxin, are identical or closely associated.

PROCEEDINGS OF THE SOCIETY OF HYGIENE OF THE SCHOOL OF HYGIENE AND PUBLIC HEALTH OF THE JOHNS HOPKINS UNIVERSITY, BALTIMORE, MARYLAND.

Regular Meeting of Monday, May 8, 1922.

The Effect of X-Ray Upon Reproduction in the Rat. By D. R. Hooker.

This is a preliminary report. The whole is designed for a particular study of the possibility of controlling reproduction and the oestrous cycle in mammals. Clinical experience has shown that the x-ray and radium may cause sterility in both men and women, and that they likewise appear to repress the oestrous cycle. It is desired to ascertain by careful animal study in how far these effects can be brought under complete control, and whether or not they exert any other deleterious consequences.

So far as the experiments have progressed, they appear to show that x-ray exposure causes:

1. A transitory loss of weight in old adults, if applied to the whole body, but that if the reproductive glands alone are exposed (in males) no loss of weight occurs. Applied to young and growing animals, the growth curve is not disturbed.
2. Small doses, i.e., four erythema doses, stimulate sexual development, when applied before sexual maturity.
3. Two erythema doses are sufficient to sterilize the male for four months or more, although actively motile sperm may be demonstrated during this period.
4. Two erythema doses may retard reproduction in the female, but larger doses are more often necessary. If applied before sexual maturity is reached, four erythema doses will not affect the subsequent reproductive history.
5. Major doses, i.e., eight erythema doses, do not affect sperm motility up to eleven days. After this the vigor and number of spermatozoa decrease, and in four weeks no more can be demonstrated. With a lesser dose (three erythema doses) the spermatozoa are feeble and few in number at the end of four weeks. The minimal sterilizing dose (two erythema doses) leaves the sperm actively motile at the end of four weeks. None of the animals so treated are successful breeders.
6. Sterilization does not affect the interstitial tissue. The growth curves of x-ray sterilized animals are entirely normal, as contrasted with those of castrated animals.

A Simple Method for Measuring the Metabolism of Small Animals.

By A. L. Meyer. From the Department of Physiological Hygiene, School of Hygiene and Public Health, The Johns Hopkins University.

An apparatus was briefly described for measuring the oxygen consumption of small animals. It constitutes a closed system and consists of two flasks, each having a capacity of approximately two liters, a small animal chamber, a by-pass and two burettes (50 c.c.), arranged in manometer fashion.

The air in the circuit is moved back and forth, by alternately raising and lowering the flasks, which contain about 3,000 c.c. of a dilute solution of caustic potash. A piece of rubber tubing connects the lower ends of the burettes. The water menisci in the burettes are at the same level at the beginning of the experiment, and are brought to the same level at the close of the experiment. The contraction in the volume of the system, incident to the absorption of the carbon dioxid and the consumption of oxygen, may, therefore, be read directly in cubic centimeters. The two burettes thus take the place of a spirometer.

During the preliminary period, while the animal is growing accustomed to the chamber, room air is aspirated through the chamber for ventilation, and the air in the circuit is directed through the bypass. The solution in the flasks is thoroughly mixed. Readings of the temperature of the air in the flasks and the air in the chamber are made, until changes in volume, as indicated by the burette, can be accounted for by changes in the temperature of the system or by changes in the barometric pressure. It is also necessary for the temperature of the animal chamber to have become constant, before the experiment proper is begun.

At the close of the experiment, the reading of the burette is corrected for any change that may have taken place in the barometer or in the temperature of the system.

*New Data on the Influence of Alcohol on the Expectation of Life in Man.** By Raymond Pearl.

The critical experimental work of Stockard on the guinea pig, and the present writer on the domestic fowl, has shown conclusively that in these forms, those individuals which throughout life receive daily dosages of ethyl alcohol by the inhalation method long outlive their non-alcoholic brothers and sisters. It is a general, indeed almost universal, supposition that the accumulated experience of life insurance companies has proved exactly the opposite to be true for man—that even the most moderate consumption of alcoholic beverages definitely lessens the normal expectation of life. The prevalence of this opinion has come about as the result of three circumstances: first, the extensive and intensive propaganda extending over many years, put forth by persons and organizations interested in bringing about the enactment of prohibitory laws; second, the fact that very few persons have taken the trouble to investigate the actuarial data on the point; and, third, the fact that life insurance companies generally discriminate in some degree (ostensibly at least) against users of alcoholic beverages.

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* Papers from the Department of Biometry and Vital Statistics, School of Hygiene and Public Health, Johns Hopkins University. No. 61.

As a matter of fact, as I pointed out some years ago after a careful investigation of the matter, the existing actuarial evidence on the point is practically worthless; is, in short, of such a character as would never be accepted as critical evidence at all by any competent scientific man Its defects arise chiefly from the following considerations:

1. Generally speaking the insurance company never knows anything about the alcoholic habits of its policyholders, except their own statements at the time of application for the policy. Now a considerable proportion of all policies are originally applied for by comparatively young men, whose habits as to alcohol use frequently change radically in subsequent years. Furthermore, since the companies tend to discriminate against alcohol users in the issuance of policies they thereby put a premium upon falsehood on the part of the applicant touching this point.

2. In the case of companies writing insurance separately for abstainers and non-abstainers there is a large, and, so far as published data are concerned, unknown class selection, quite apart from the alcoholic considerations.

Lack of space forbids detailed consideration of the existing actuarial data. I hope shortly to undertake this in another place.

What is needed is a body of data in which the following items are accurately known: (*a*) alcoholic habits throughout life in detail, (*b*) age at death, (*c*) race stock, (*d*) occupation, (*e*) sex, (*f*) cause of death.

Exactly this sort of material I have been able to collect from the Family History Records of this laboratory. These Family History Records are elaborate and detailed original pedigrees collected by a staff of trained eugenic field workers. The collection now comprises detailed records of the life history of more than 75,000 persons living or having lived in and near Baltimore. Especial emphasis has been laid throughout the work on getting, wherever possible, exact and detailed records as to alcoholic habits throughout life. Some months ago I had extracted from the Records all the cases then available showing the items specified above. Some aspects of these I wish to present in a preliminary way in Tables I and II. The numbers, while not large in an actuarial sense, are large enough to give reasonably small probable errors in all the series, except the heavy and steady drinking females. There the number is insufficient upon which to base a survivorship table. No individual is included who died before age 20, for obvious reasons.

TABLE I.

Survivorship distributions for white persons of the following drinking habits: (a) total abstainer, (b) moderate and occasional drinker, (c) heavy or steady drinker. 1,000 supposed entering at age 20 in each class.

Age.	Male.			Female.	
	Abstainer.	Moderate.	Heavy.	Abstainer.	Moderate.
20.....	1,000	1,000	1,000	1,000	1,000
25.....	973	988	985	967	971
30.....	923	946	951	925	902
35.....	887	878	887	867	878
40.....	820	821	817	782	854
45.....	752	774	692	724	790
50.....	698	714	602	674	741
55.....	640	619	483	591	629
60.....	586	539	406	504	561
65.....	477	452	337	419	444
70.....	365	357	267	313	366
75.....	234	250	167	218	283
80.....	135	179	105	120	180
85.....	63	92	57	65	117
90.....	45	54	21	13	59
95.....	27	24	5	5	20
100.....	9	3	0	0	0

TABLE II.

Mean age at death of persons of different drinking habits.

Drinking habits.	Males.		Females.	
	N.	Mean. (Years.)	N.	Mean. (Years.)
Total abstainers........................	222	60.68±.85	399	58.44± .62
Moderate and occasional................	336	60.95±.69	205	61.48± .91
Heavy or steady........................	389	56.41±.59	18	45.00± 1.9

From these data, representing a racially and occupationally homogeneous group, in so far as the different drinking categories are concerned, we may conclude:

1. That there is no indication in either sex that the moderate and occasional consumption of alcoholic beverages adversely affects expectation of life. In the females the moderate and occasional group gives a mean value 3.04 ± 1.10 years *higher* than the abstainer group, a difference possibly, but not very probably, statistically significant.

2. That the individuals in the heavy or steady drinking group have a *significantly lower* expectation of life than those in either of the other groups.

While the whole number involved (1,569) is still statistically small, it is believed that the data are more critically accurate, in the sense of the laboratory experimenter, than any which have hitherto been available on this problem.

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ON THE MODE OF ACTION OF DYESTUFFS ON BACTERIA.*

By F. P. GAY and T. D. BECKWITH.

(Received for publication April 18, 1922.)

Chemotherapy in its broadest aspects has made enormous progress in recent years. One of the first suggestive possibilities of chemotherapy arose in the observations of Koch and of Stilling (1890) on the bactericidal effect of dyestuffs on bacteria. Observations along this line continue to interest experimenters, but practical results in the treatment of bacterial diseases by means of dyestuffs have been attained only within very narrow limits. It must be admitted that recent observers seem to have demonstrated a very distinct favorable action by dyestuffs on localized infections to which the material can be directly applied. Thus we have favorable results in treating wounds reported by Massie, Drummond, Carslaw, Ligat, Pilcher and Hall, and Feiler. The dyes most frequently used for this purpose are brilliant green and the acridine compounds. Chancroid may be controlled by the local application of carbol fuchsin and methylene blue according to Queyrat, and brilliant green has been found useful in checking the extension of erysipelas by Adams. Gonorrhoea has been favorably affected by local application of methylene blue (Dorland) and of acriflavine (Davis and Harrell).

The treatment of closed serous cavities, such as the meninges and the pleura, by dyestuffs must still be regarded as of problematical value. Favorable results have been claimed in joint infections by lavage with gentian violet, by Churchman. Tuberculous empyema has been treated in a similar way by Waters, and streptococcus empyema by Major, but as the latter correctly remarks, no control cases with simple aspiration were employed. Kolmer and associates (1,

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* From the Department of Bacteriology and Experimental Pathology, University of California.

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2, 3, 4) in a recent series of articles has suggested the usefulness of certain chemotherapeutic agents other than dyestuffs in the treatment of infections of serous surfaces, at least in experimental animals, where a natural resistance to the infection in question is present, or the virulence of the bacterium is low. The results of the senior author (Gay and Morrison) in treating localized streptococcus empyema with dyestuffs gave little encouragement for the treatment of the more virulent types of infections.

It seems still less likely that generalized infections and infections in remote local areas that must be reached through systemic injections will be markedly benefited by dyestuffs. We have here to consider a number of factors that do not prevail, or at least prevail to a less degree in the treatment of local, accessible infections. We must consider more definitely the toxicity of the material employed for the treated animal; the reduction of the dyestuff in the animal body with consequent loss of its bactericidal power; and finally the actual attainment of the local point of infection. The latter difficulty may be overcome when dyestuffs that are excreted through the urine or bile are employed, and if we desire to affect bacteria resident in either one of these localities. This possibility is of extreme importance as bearing on not only active infections, but on the carrier condition. It has been shown experimentally that certain dyestuffs, for example acriflavine (Browning and Gulbransen) and gentian violet (Churchman and Herz), endow the blood of animals with bactericidal property for at least a period of time, but very little effect has been obtained in the actual treatment of generalized experimental infections by this means (Browning and Gulbransen, 2, 3; Tubby and Ferguson; Petroff; Neufeldt and Schiemann). The apparently favorable effect on coccidioidal granuloma by Brown and Cummins, and on trench fever by Byan, Dimond, Sorapure and Wilson by similar methods must be regarded, we believe, as inconclusive. The treatment of infections of the genito-urinary tract through the elimination of acridine and thiazine dyes has at least become possible by the work of Davis, and similar suggestive results have been obtained with new fast green, in experimental typhoid carrier rabbits, by one of us (Beckwith).

In reviewing rather carefully this field of germicidal dye action and of possible dye therapy, it seems to us that very little progress has been or will be made simply by purely empirical attempts to cure experimental or natural infections by administering dyestuffs that are found to kill bacteria in the test tube. Much of the work already done has been of this nature. It does seem important, however, whatever the practical outcome may be, to obtain all the information possible on the mode of action of dyestuffs on bacteria, and further, on the fate of dyestuffs in the animal body. It is with the first of these considerations particularly in mind that we bring together at this time certain of our experimental results.

Many of the studies of the action of dyestuffs on bacteria have been casual rather than systematic. A few comprehensive attempts to study the whole question have been made, notably the one by Eisenberg, and at least two working hypotheses of the mode of action of dyestuffs on bacteria have been advanced; namely, the one proposed by Eisenberg and the more concrete, but less substantiated outgrowth of this hypothesis recently suggested by Langer. The casual nature of the experiments with the effect of dyestuffs on bacteria, the variations in the technique employed, and like discrepancies have left considerable question as to the essential facts so far obtained. Some of these facts, however, are undoubted and may be briefly summarized at this point.

In the first place there is a general consensus of opinion that Gram positive bacteria are more readily inhibited or killed by dyestuffs than are Gram negative bacteria (Churchman, 2; Eisenberg; Kolmer; Woody and Yagle, 5). This greater susceptibility of the Gram positive organisms would apparently be directly associated with the greater permeability of this group of micro-organisms (Eisenberg, Brudny). In addition to the general hypersusceptibility of Gram positive organisms, there are evidences in the work of all those investigators, beginning with Roszahegyi, who have studied any number of bacteria and of dyestuffs, that there is at times a definitely specific action, that is to say, a given dyestuff will affect one Gram positive organism more than another, whereas a second dyestuff will affect the second organism more than the first.

Certain general facts relate to the dyestuffs themselves. It is generally admitted that the basic dyes are more bactericidal than the acid dyes (Dreyer, Kriegler and Walker; Isabolinsky and Smoljan). Kriegler has shown that the rosaniline and thiazine dyes increase in lethal action with basicity, whereas in the acid dyes they increase with the acid radicle. The addition of a base will increase the action of dyestuffs (Graham-Smith; Prowazek; Traube). Browning and Gulbransen (4, 5), and Beckwith have shown that the decrease in true acidity, as measured by the hydrogen-ion method, will increase the effectiveness of acridine dyes.

Bacteria have been shown to have a distinct power of reducing dyestuff in the process of their growth (Nicolle, Gordon, Marino). The whole question of vital staining of bacteria is a disputed, although interesting one. It would appear very doubtful whether living yeast cells or bacteria are actually stained (Eisenberg, Nicolle). It would seem that although stains may be found in bacteria and those bacteria actually be proved to be living, the fact of their staining is an indication that they are in the process of disintegration. At all events in this connection, a method of staining has been suggested by Proca, which has been successfully utilized in the differentiation of living and dead bacteria. These results have been confirmed by Stemschneider and somewhat modified and improved by Kayser. We can confirm the general fact of a differentiation between living and dead bacteria as incidental to some of our own work and have obtained better results by Kayser's method. We agree also with Stemschneider that streptococci give irregular results. The nature of the cell wall of the bacteria would seem to be the important factor in the entrance of the dyestuff into the bacteria and its consequent action on it. The actual passage through the cell wall may depend on variations in surface tension (Traube; Hansen). At all events a greater permeability for dyes has been shown in the Gram positive organisms by Eisenberg and by Brudny, and certainly the nature of the dyestuff itself, probably the physical nature rather than the chemical structure, affects both the entrance and the residence of the dyestuff inside the bacterial body. There is some suggestion, according to Eisenberg, that the lipoid solubility of dyestuffs is of great importance in determining the entrance through the cell wall. There are, however, examples of lipoid soluble dyes that do not affect bacteria and of lipoid insoluble dyes that are highly bactericidal. Of greater importance still, in all probability, is the degree of dispersion or the colloidal character of the dye. Eisenberg has shown that Gram negative organisms are relatively impermeable for electrolytes, whereas the Gram positive organisms have a greater absorbing affinity, particularly for high molecular dyes. He emphasizes the importance further of the degree of dispersion, perhaps in some way associated with the lipoid solubility of the dyes, as affecting their action on the bacteria. Langer has offered a still more comprehensive, though distinctly less documented conception of the mode of action of dyestuffs on bacteria.

According to Langer, within certain limits the greater the dispersion of a given dye, the greater the disinfecting power. The wall of the bacterium is like a net and molecules of a certain size are admitted in accordance with the dimensions of the meshes of this net. Once the molecule of dyestuff has passed through the net meshes, it is immediately swollen by the action of electrolytes inside the cell. If, on entrance, it was sufficiently small, it may still pass out, even after increasing in size; if, however, it was large enough barely to pass through the meshes, it can not escape after its increase in size within the bacterium, and remains there to produce its lethal effect. By means of this conception Langer would explain the varying combinations between dyes and bacteria, which occur both within and without the body. The addition of serum to the majority of dyestuffs causes an increase of dispersion to the point of precipitation, and the disinfecting action is decreased or lost. This is the usual result when dyestuffs are introduced into the body. In the second instance there is no increase in the size of the molecules. This is what usually happens in the use of chemical substances like salvarsan or optochin. The third possibility occurs where the colloidal condition is increased to a desirable extent for penetrating bacteria, but not sufficiently to result in precipitation of the dye with loss of bactericidal power. This is recognized to occur with the acridine dyes on the addition of serum

Personal investigations.

Our experiments, to be reported at this time, deal primarily with the effect of blood serum on the bactericidal action of dyestuffs, and in this way have a direct bearing on the possibilities of dye-therapy. As we have already stated, it is well recognized that protein solutions, particularly serum, decrease the bactericidal activity of the majority of dyestuffs, and this decrease is usually associated with visible flocculation and decolorization of the dyestuff in question. It is evident from the work of one of us (Beckwith, 2) that dyes vary notably in their susceptibility to change by body fluids and body tissues, and that with a susceptible dye different tissues show distinct differences in their reducing power. This reduction in bactericidal power by body fluids is undoubtedly the most important factor in the failure of dyestuffs to work effectively against bacterial infections in the body. There is one notable exception to this depressing action of protein on dyestuffs, which is furnished by the acridine compounds, as shown in test tube experiments. Under certain conditions these dyes not only do not suffer a depression of bactericidal effect, when serum is present, but are actually enhanced in their destructive power for micro-organisms. This, together with the fact that the acridine dyes are relatively non-toxic for animals; that they are rather widely distributed throughout the tissues, as shown by their coloration; and that they are eliminated both through the urine and the bile, and endow the blood serum at least with temporary bactericidal effect, has resulted in numerous investigations of this group of compounds.

We have adopted particularly for our study three dyes which are typical from the standpoint under investigation. (1) *Acriflavine* (Heyl Laboratories, Inc.) is one of the acridine dyes which is relatively non-toxic for animals; highly destructive for all varieties of bacteria; more bactericidal in the presence of blood serum; and not reduced appreciably in the animal body, so far as color appearances can be trusted. (2) *Methylene Blue* (medicinally pure, Grübler), moderately bactericidal; non-toxic for animals; slowly reduced in the body; slightly or not at all altered in bactericidal effect in vitro by presence of blood serum. (3) *New Fast Green* (Society of Chemical Industry, Basle), highly bactericidal in broth cultures; markedly reduced in effectiveness in the presence of blood serum; under certain conditions, discussed below, fatal for animals, but ordinarily not so; temporary coloring of animal tissue, particularly when the compound becomes toxic.

The micro-organisms particularly investigated in connection with these dyestuffs have been the Gram negative *B. typhosus* and the Gram positive *Streptococcus hemolyticus.*

Technique employed. The titer limits of the various dyestuffs studied have been determined under varying conditions for the micro-organisms in question, by adding to a solution of the dyestuff in peptone water or broth, in a volume of 1 c.c., small amounts of a 24 hour infusion broth culture. In the case of the *Streptococcus pyogenes* (strain "H," passage culture, Gay and Stone), .1 c.c. of the fresh culture was usually added to each tube containing 1 c.c. of the varying dilutions of dye. In the case of *B. typhosus* utilized (strain 3 B.), .01 c.c. in .1 c.c. volume of fresh broth culture was used. These mixtures of dyestuffs and bacteria, with or without the presence of various protein or other materials, were incubated at body temperature, streaks from the various tubes were made on blood agar (*Streptococcus*), or plain agar (*B. typhosus*) at intervals under 24 hours; in all events, after 24 hours' incubation the presence or absence of living bacteria was thereby determined in each tube, and the greatest dilution of dyestuff yielding sterile cultures at this time was accepted as the titer limit.

The relative susceptibility of Gram negative, as contrasted with Gram positive, micro-organisms may be indicated by an experiment with the relatively weakly bactericidal methylene blue.

TABLE I.

Bactericidal titer of methylene blue (M. P.) in peptone solution and in inactivated (56° C.) rabbit serum for 0.01 c.c. (in 0.1 c.c.) of 24-hour broth culture of various micro-organisms.

	In peptone.	In serum.
A. *Gram Positive:*		
B. anthracis	1–100,000	1– 50,000
B. hoffmanni	1–100,000	1–100,000
Staph. aureus	1–100,000	1– 50,000
Streptococcus pyogenes	1– 80,000	1– 10,000
B. smegmatis	1– 1,000	1– 1,000
B. subtilis	> 1– 1,000	> 1– 1,000
B. *Gram Negative:*		
B. prodigiosus	1– 10,000	1– 1,000
B. mucosus capsulatus	1– 1,000	1– 1,000
B. proteus	> 1– 1,000	> 1– 1,000
B. pyocyaneus	> 1– 1,000	> 1– 1,000
B. typhosus	1– 500	1– 750
Sacch. cerevisiae	1– 200	1– 800

(The sign > equals "less than.")

It is here evident that, with exceptions, the Gram positive bacteria are more readily destroyed by methylene blue than the Gram negative. With a single exception the presence of serum leaves the bactericidal titer unchanged or slightly lowered.

A comparison of the relative bactericidal effect with and without serum of the three dyestuffs we have employed, in more detail, upon *B. typhosus* and *streptococcus* shows the following bactericidal effect:

This table illustrates again the difference in susceptibility between a Gram positive and a Gram negative micro-organism, and shows the depression or adjuvant effect of serum, in accordance with the dye employed.

TABLE II.

Bactericidal titer of three dyestuffs diluted in broth or serum. In this case 0.1 c.c. of culture of B. typhosus or of Streptococcus pyogenes.

	Broth.	Serum.
Acriflavine:		
B. typhosus	25,000	100,000
Streptococcus pyogenes	160,000	640,000
Methylene Blue:		
B. typhosus	500	750
Streptococcus pyogenes	80,000	10,000
New Fast Green:		
B. typhosus	50,000	1,000
Streptococcus pyogenes	100,000	1,000

It will be noted from our remarks under technique that we have utilized somewhat different doses of the two organisms with which we have worked. Inasmuch as the dose of typhoid bacillus employed was smaller than that of streptococcus, in most experiments, and yet the action of the dyestuff distinctly less on the typhoid bacillus than on the streptococcus, the differences we wish to bring out between the Gram positive and the Gram negative organisms would have been enhanced, if precisely the same dosage of each had been employed. The question naturally arises, however, as to the relation between the number of bacteria to be destroyed and the concentration of the dyestuff. It is found that the bactericidal titer varies directly, though not uniformly, with the number of bacteria present (Table III).

Varying amounts of a 24-hour infusion broth culture, ranging from 10 c.c. to 0.01 c.c., were either concentrated by centrifugalization, or were diluted so as to be contained in a total volume of 1 c.c. These amounts were then tested, in series, with varying concentrations of acriflavine, diluted in broth, and the final bactericidal titer in 24 hours was as follows:

TABLE III.

For 10 c.c. culture *Streptococcus* H	1– 8,000
For 1 c.c. culture *Streptococcus* H	1– 64,000
For 0.1 c.c. culture *Streptococcus* H	1–128,000
For 0.01 c.c. culture *Streptococcus* H	1–256,000

Similar results have been obtained with various doses of typhoid bacillus and new fast green. We have also found that the rapidity of action of acriflavine on a given dose of streptococcus varies roughly with its concentration.

In this connection the previous remarks of one of us (Gay and Morrison) should be noted. We found in our studies on a number of dyestuffs that streptococcus pus was sterilized by a greater dilution of acriflavine than was a broth culture of the same organism. This statement apparently differs from the findings of Browning and Gulbransen (2), Hewlett and Dakin and Dunham, who have noted that pus is slightly inhibitory in its action, though all authors admit that serum increases the action of acriflavine. We have attributed these differences to the fact that in our experiments the pus contained only a small percentage of the number of organisms present in the broth cultures. Indeed in some of our experiments, where large numbers of organisms were present in the pus, no distinct increase in bactericidal action in pus over broth was noted.

In this connection it is of interest to note that acriflavine, repeatedly treated with concentrated rabbit pus, either sterile from normal animals or containing streptococci, does not lose appreciably in bactericidal titer. We have further been surprised to find that when acriflavine is added in concentrated solution (10 per cent.) to pure rabbit serum, and a dense precipitate formed and removed, the supernatant fluid has lost very little of its original germicidal power. Small amounts of streptococci (sediment of 1 c.c. of a broth culture) may be added several times to acriflavine without reducing the dye titer, but when large amounts of culture (15–20 c.c., three times) are added, the titer is reduced to about one fourth its original power. These are matters that will be again referred to in our final discussion.

We may now consider more attentively the increased or decreased effectiveness of dyestuffs, in the presence of serum. The addition of serum to new fast green is accompanied by visible precipitation and decolorization of the dye. This is accompanied, as stated, by decrease in bactericidal power. Methylene blue, when added to serum, is slightly decolorized and the bactericidal effect slightly altered. Acriflavine, in certain concentration, in serum gives a distinct precipitate, which is at least partly dissolved on successive further dilutions in serum or water, and the bactericidal effect not only is not decreased by precipitation, but is increased by the presence of serum. As we have already indicated, a most attractive theory of the mode of action of dyestuffs on bacteria has recently been advanced by Langer, who would explain the variations obtained by (1) the size of the pores in the bacterial envelope, and (2) the size of the molecules of the dyestuff in question and its possibility of entering these pores and remaining subsequently inside the bacteria, owing to further increase in molecular size. This theory finds no contradiction in the facts that we have stated to the present point. New fast green rapidly penetrates the bacterial wall when no serum is present, and therefore destroys the micro-organism. In the presence of serum the dispersion of new fast green is increased to the point of flocculation, and the dyestuff is unable to pass through the meshes of the net work of the bacterial wall. Acriflavine, on the other hand, passes into bacteria in the absence of serum, readily, and remains sufficiently to be very bactericidal. When the size of its molecule is increased by some combination with serum, it remains even better within the bacteria and is therefore more bactericidal. Langer verifies his hypothesis indeed by a comparison of the bactericidal effect of ordinary acridine (Acriflavine ?), which, although markedly bactericidal, is distinctly less so than its higher molecular compound, Flavizid, which is produced by the addition of methyl groups. One of our experiments would seem to indicate, on the contrary, that the acridine compounds, at least, do not increase in bactericidal power with their molecular weight:

TABLE IV.

The bactericidal titer of various acridine compounds for B. typhosus, in accordance with their molecular weight.

Compound.	Molecular weight.	Titer in broth.	Titer in serum.
Proflavine	250 (Browning)	10,000	100,000
Acriflavine	264 "	25,000	100,000
Acridine orange	301 (Green)	1,000	> 1,000

As already stated, one could explain the increased action of acriflavine on the addition of serum as due to the formation of a compound of greater colloidal properties. If this were true it should be possible also, by the addition of other protein and of non-protein colloids, to increase the action of acriflavine, as is the case when serum is used. Our experiments in this direction are summarized in the following experiment (1), together with its explanatory technical notes:

Experiment 1. Bactericidal titer of acriflavine, in solutions of various plant and animal proteins and non-protein colloids, against

B. typhosus.

Materials employed.

Whey of cow's milk, obtained by adding rennin solution to skim milk and removing curd. The fluid was heated to 56° for twenty minutes.

Beet juice was obtained by grinding cut beets in sand, with a sterile mortar and pestle, and extracting with sterile water. This extract was heated to 56° before using, and then centrifugalized and the supernatant fluid poured off. The extract gave a copious precipitate with alcohol.

Bean extract. Ran small white beans through a meat chopper and extracted with sterile distilled water for 18 hours in the ice box. The fluid was decanted and heated to 56°. It gave a copious precipitate with alcohol.

These solutions were used as diluents of the stock aqueous (1%) acriflavine solution.

Protein or Colloid.	Bactericidal titer of acriflavine.
Horse serum 56°	100,000
Human ascitic fluid	100,000
Human hydrocele fluid	100,000
Human thoracic fluid	100,000
Peptone water check	25,000
Skim milk	1,000
Whey	10,000
Beet juice	> 1,000
Bean extract	10,000
Gelatine 3% in distilled water	10,000
Gum acacia 3% in distilled water	1,000
Cocoanut milk	> 1,000

In all instances check tubes with the protein or colloid in question, but without acriflavine, gave abundant growth of *B. typhosus.*

In these instances it is evident that the only protein solution which increases the action of acriflavine is animal serum or its analogues. The other proteins and colloids actually decrease the bactericidal titer of the dye.

Similar experiments were carried out with egg white, and at first glance it appeared that this protein substance added to the bactericidal effect of acriflavine in a surprising manner. Controls, however, show that egg white is bactericidal in 50 per cent. solution, for both *B. typhosus* and the streptococcus. This point was noted many years ago by Wurtz. Both the streptococcus and the typhoid bacillus grow in a 25 per cent. solution of egg white, however, but there is no markedly increased activity in acriflavine to which this dilution of egg white has been added. The bactericidal titer of acriflavine in 25 per cent. egg white and in broth was identical for streptococcus, but one tube higher in the egg-acriflavine for *B. typhosus.* This latter result, in view of the fact that egg white is at least to some degree in itself bactericidal, would appear to be a cumulative effect rather than an adjuvant action. It is further evidenced that the egg white differs from serum in that the protein fractions (ovoglobulin and ovalbumin) depress acriflavine action, whereas serum albumin, at least, increases it. (See Table X.)

The question naturally arises whether the particular percentage of protein, that is to say, the degree of dispersion of the solution, might not be at the optimal point in serum, and at some less favorable point in the other proteins and colloids employed. In order to convince ourselves of this effect, we have carried out further experiments, in which various percentages of colloids were employed. (See Tables V and VI.)

TABLE V.

Effectiveness of acriflavine in sterilizing EQUAL VOLUMES *of a broth culture of Streptococcus pyogenes "H," in varying percentages of aqueous gum acacia in broth.*

Acriflavine in:	Titer.
Plain broth	40,000
0.6 per cent. gum acacia broth	40,000
1.5 per cent. gum acacia broth	20,000
3.0 per cent. gum acacia broth	10,000
6.0 per cent. gum acacia broth	5,000

Controls in the varying percentages of gum acacia broth, without the acriflavine, grew perfectly.

This experiment, with various percentages of gum acacia broth, shows that increasing the colloid from 1.5 per cent. upward results in a steady decrease in the bactericidal titer of acriflavine added to it, whereas in a 0.5 per cent. solution it is the same as in plain broth.

In another experiment the protein percentage of rabbit serum, casein and peptone was determined from the nitrogen content, as given by the Kjeldahl method, by multiplying by 6.25, and all were reduced by dilutions with physiological saline solution to the same protein ratio. Care also was taken to adjust the pH to a similar figure, as it is known that under certain conditions the degree of acidity in any dyestuff affects the germicidal property.

Experiment 2. 1 gram of purified casein was dissolved in 8 c.c. of $\frac{N}{10}$ NaOH. 9 c.c. of physiological saline were added and then 1.5 c.c. of $\frac{N}{10}$ HCl. Physiological saline added to 20 c.c. This solution was sterilized in the Arnold. The pH was 6.9. The protein estimate from Kjeldahl was 5.2 per cent. Inactivated rabbit serum pH 7.4. Protein = 6.3 per cent. 5 per cent. peptone solution in water pH 7.2. Protein = 4.1 per cent.

The first two solutions were reduced by the addition of saline to 4.1 per cent. protein and the bactericidal titer of acriflavine in these respective solutions determined.

Titer of acriflavine for 0.1 c.c. culture Strept. "H" diluted in	
Peptone water 4.1 per cent. Protein	160,000
Casein 4.1 per cent. Protein	160,000
Rabbit serum 4.1 per cent. Protein	640,000

Controls without acriflavine positive.

This experiment demonstrates clearly that whereas acriflavine is increased in bactericidal property in serum over peptone solution, casein with the same amount of protein substance, as determined by the nitrogen content, stands at the same point as peptone.

If it were true that serum acts as an adjuvant to the bactericidal effect of acriflavine by increasing dispersion, we should expect that up to a certain point at least a further increase of the serum molecule might still further enhance this effect. Serum may be diluted in distilled water and then heated to various degrees without coagulation, but the dispersion is increased, as evidenced by increasing opacity in the solution. The adjuvant effect of serum heated to 56°, as compared to serum heated to higher degrees, on the bactericidal effect of acriflavine is illustrated in the following table (VII), where it is found that the further increase in size of the serum molecule decreased its adjuvant effect on acriflavine.

It is evident from these experiments with acriflavine that we have found no corroborative evidence for the theory that the adjuvant effect of serum on acriflavine is due simply to increased colloidal dispersion. There is on the contrary considerable negative evidence against its being a simple phenomenon of this sort. Objections, of course, could be made to such experiments as those in our last table on the ground that the dispersion proceeded too far toward the stage of flocculation for the most effective action to be insured.

TABLE VII.

Rabbit serum was diluted with equal parts of physiological saline solution or water, and portons heated for 15 minutes to 56°, 75°, and 85° C. The two higher temperatures turn the diluted serum opalescent and then opaque, but there is no precipitation.

Bactericidal titer of Acriflavine diluted in:	Streptococcus 0.1.	B. typhosus 0.01.
Broth	160,000	25,000
Rabbit serum 56° C.	320,000	80,000
Rabbit serum 75° C.		20,000
Rabbit serum 85° C.	40,000	20,000

When we turn to our experiments with new fast green, other and in some respects more interesting material along similar lines becomes available. It will be recalled that when serum is added to new fast green, flocculation occurs, accompanied by decolorization of the dyestuff. Here obviously the size of the particles and their insolubility would render germicidal action less efficient. It is, however, possible to prepare new fast green in a colloidal condition. Many simple electrolytes will change an aqueous solution of this dye to a gel. New fast green is readily soluble in a concentration of 1 per cent. in hot distilled water. If to such a solution varying amounts of electrolyte are added, the solution at body temperature, or more slowly at room temperature, becomes gelatinous. This physical change takes place more rapidly after boiling. This gel may be shaken into a semi-fluid condition. Solutions of new fast green below a certain concentration remain soluble even in the presence of an electrolyte. The following table shows the percentage of new fast green necessary to produce the gel condition in the presence of a 1 per cent. solution of certain electrolytes:

TABLE VIII.

Percentage of new fast green necessary to form a gel in the presence of 1 per cent. of various electrolytes.

Electrolyte.	Percentage of new fast green.
Distilled water	No gel at saturation
Magnesium sulphate	1 per cent.
Calcium chloride	0.25 per cent.
Sodium chloride	0.125 per cent.
Potassium nitrate	0.031 per cent.

There are certain substances used in colloidal industrial processes which are useful in keeping precipitates from appearing in solutions.

These are known as stabilizers or peptizers. Various of these materials were added to a solution of new fast green gelled by the presence of potassium nitrate, the most active electrolyte in producing this result, and it is evident that dextrose, potato starch, dextrin and glycerine, in the increasing order named, prevent the gel from forming up to a certain point.

TABLE IX.

Prevention of gel formation in new fast green by a stabilizer in the presence of 0.5 per cent. potassium nitrate.

Stabilizer.	Percentage of N. F. G. in which gel forms.
None	0.062 per cent.
.125 per cent. potato starch	0.125 per cent.
5 per cent. dextrose	0.125 per cent.
5 per cent. dextrin	0.25 per cent.
50 per cent. glycerine	Trace at 0.5 per cent.

Flocculation in new fast green by means of serum may be due to the next stage in the dispersion of the dyestuff, produced by the electrolyte in serum. At all events it can be shown that glycerine at least will delay the flocculation of new fast green in contact in serum, which might be a matter of possible practical applicability in the actual treatment of infection with this material.

We have already referred to the work of one of us (Beckwith), which shows that under certain apparently unpredictable conditions new fast green is toxic for rabbits when injected intravenously. In the light of our results with the formation of a gel of new fast green by an electrolyte, it appears evident that the anaphylactoid symptoms described in this connection are due to embolism of vessels by a gel produced in the injected dyestuff. Whenever death or severe anaphylactoid symptoms are produced in this way, there is a diffuse coloration of the animal which does not occur when the dye is non-toxic. This obviously suggests the work with colloidal dyes of Schuleman and Evans. Although rabbits may die acutely when given intravenous injections of new fast green, presumably in gel state, the question naturally arises whether the dyestuff is in itself more toxic, apart from the mechanical embolic effects produced. New fast green is even more fatal for guinea pigs than it is for rabbits, and the question may be definitely answered as in the following experiment:

Experiment 3. A 1 per cent. solution of new fast green in hot distilled water was prepared. When further diluted in equal parts of boiling physiological saline (0.85 per cent.), it gelled on cooling. Boiling distilled water in equal parts produced no physical change in the dye. The saline gel preparation (0.5 per cent. of dye) could be made semi-fluid by shaking and was injected in decreasing doses, diluted in saline, into the peritoneal cavity of a series of guinea pigs. A similar series of animals was given the corresponding doses of the aqueous sol dilutions of the dye. The amount injected was estimated in accordance with the body weight.

Guinea pig.	Weight.	Dose of dye per 100 grams.	Result.
1..........	350	0.5 c.c. 0.5 per cent. aqueous sol.	Dead 18 hours
2..........	400	0.2 " " " " " "	" " "
3..........	350	0.1 " " " " " "	Recovered
4..........	550	0.05 " " " " " "	Recovered
5..........	300	0.5 c.c. 0.5 per cent. saline gel	Dead 18 hours
6..........	250	0.2 " " " " " "	" " "
7..........	500	0.1 " " " " " "	Recovered
8..........	350	0.05 " " " " " "	Recovered

This experiment shows conclusively that the new fast green in gel state is not more toxic for guinea pigs than the sol condition of the same dye.

In more direct interest with our present investigation it may also be shown by titration that the sol and gel state of new fast green have the same bactericidal limit of dilution for the streptococcus. Thus in an experiment with three preparations of new fast green in which the dye was added in the proportion of 0.5 per cent. to cold water (1), boiling water (2), and boiling saline (3), the result in cooling was found to be 1 = partial solution with some precipitate; 2 = complete solution; 3 = gel. On further dilution of 1 and 2 in water and 3 in saline solution, the titer limits of destruction of equal volumes (1 c.c.) of a broth culture of our Streptococcus "H" were found identical, namely 1–32,000.

In another experiment with *B. typhosus* it could be shown that the addition of 1 per cent. gelatine to new fast green actually decreases the bactericidal effect of this dye, and similar results were given with gum acacia.

We have then no parallel to the adjuvant action of serum on acriflavine in the use of other colloids. It is apparently an exceptional, but regular effect, and it remains to be seen what more can be learned as to its intimate mechanism. If serum is flocculated by means of ammonium sulphate in the ordinary fashion (Hartley), it may be shown that the euglobulin and albumin fractions, when added in solution to acriflavine, increase its effectiveness and that pseudoglobulin decreases it, as shown in the following table:

TABLE X.

Bactericidal titer of acriflavine in the presence of protein fractions of horse serum with B. typhosus and Streptococcus "H," each 0.01 c.c.

	With B. typhosus.	With Streptococcus.
Euglobulin	1– 75,000	1–1,000,000
Pseudo-globulin	1– 10,000	1– 10,000
Serum albumin	1–100,000	1– 500,000
Peptone water	1– 25,000	———
Whole serum	1–100,000	———
Physiological saline	———	1– 75,000

It is perhaps of interest to note at this point that the exact reverse of this is the fact when these fractions are added to phenol, in an attempt to influence its bactericidal property; that is to say, the pseudoglobulin leaves the phenol unaffected, and the serum albumin and euglobulin fractions decrease the bactericidal effect.

TABLE XI.

Bactericidal titer of phenol in presence of protein fractions of horse serum with B. typhosus 0.01 c.c.

Euglobulin	1–500
Pseudoglobulin	1–750
Serum albumin	1–500
Peptone water	1–750

When bacteria are treated with inactivated rabbit serum, whether of a normal animal or of a specifically immunized animal, and the serum is removed after contact for an hour or two, there is no increase in the bactericidal effect when they are added to increasing dilutions of acriflavine. In other words there is no evidence that the serum unites with the bacteria so as to render them more easily acted upon by the dyestuff. In a few exceptional instances, it was apparently demonstrated that serum does in some way unite with both the micro-organisms we have studied, so as to render them less susceptible to the action of methylene blue, that is to say, similar to the effect produced when the excess of serum is left surrounding the bacteria. In the majority of experiments no difference in susceptibility of serum-treated bacteria to methylene blue was encountered.

Discussion and conclusions.

We have attempted to evaluate certain of the factors that influence the bactericidal action of dyestuffs, both within and without the body.

In the first place we have confirmed and extended the observations of others which point to the fact that Gram positive organisms are more readily destroyed by dyestuffs in general than are Gram negative organisms. Both facts are doubtless due to some differences in their structure and permeability (Eisenberg). The basis, however, of this fundamental difference between the two large groups of organisms still eludes us, and is doubtless capable of further elucidation. We have been interested more particularly in some factors that affect the action of dyestuffs in the body. We know that the factors that militate against the successful destruction of bacteria in the body are briefly (1) the toxicity of the dyestuff for the host, (2) the alteration and consequent loss of bactericidal power in the dyestuff through the action of body tissues and fluids, and (3) the inaccessibility of the particular foci of infection concerned. Blood serum represents in many respects the tissue fluid that most affects the action of dyestuffs in the body, and we have, therefore, been interested particularly in the effect of blood serum on the action of dyestuffs in vitro. The dyes that we have investigated represent the various possibilities of interaction with serum; namely, new fast green, a dye which is inhibited in action by serum; methylene blue, a dye which is little or not at all affected by serum; and acriflavine, a substance, the bactericidal activity of which is increased in the presence of serum.

Langer's recent theory of dyestuff action is an ingenious one and would explain the grade of activity as due entirely to an optimal degree of dispersion of the dyestuff. This theory offers explanation, why some compounds of the acriflavine series of greater molecular size are more active than others, and also would apparently explain why acriflavine is more active in serum than in broth. Our own experiments do not, however, confirm this hypothesis, since we find first that some acridine compounds do not increase in bactericidal power with molecular weight, and that they are not increased in the presence of any other protein or colloid material than serum. In fact, most of these other substances decrease the action of all the dyestuffs we have utilized. The adjuvant action of serum is almost equally present in its euglobulin and albumin fractions. The two fractions, on the contrary, depress the germicidal activity of phenol. The pseudoglobulin fraction appears to be without effect on either germicide.

New fast green can be employed either in a sol or gel condition, the latter condition being produced by the action of an electrolyte; and they are identical in their toxicity for animals, if we obviate the mechanical effects of intravenous injection, and in their destructive power for bacteria. Although the gel condition is induced by the presence of an electrolyte, there are stabilizers or peptizers of varying power, such as glycerine, which by their presence may inhibit this sol to gel change. This effect may take place either with serum absent or present. It is possible that these factors may be of importance in future developments in the field of chemotherapy.

Serum apparently does not act directly upon bacteria so as to render them subsequently more or less susceptible to the action of dyestuffs, and it can not be shown that there is any great combining power between dyestuffs and bacteria themselves, or at least it may be stated that there is no union in variable proportions between the bacteria and dyestuffs, and that the dyes are not used up rapidly in their destruction of bacteria.

There is no evidence that the action of dyestuffs on bacteria is analogous to the photosensitizing action of fluorescent dyes on red blood cells. The latter action is inhibited by serum, whereas, as we have seen, some dyes are increased in their effect on bacteria by serum and furthermore, the most typical flourescent dye, eosin, has relatively little bactericidal effect.

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AN INTRACUTANEOUS TEST FOR THE VIRULENCE OF DIPHTHERIA BACILLI IN FIELD CULTURES.*

By JOHN N. FORCE and MARGARET I. BEATTIE.

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I. Introduction.

In 1887, soon after his discovery of the diphtheria bacillus, Loeffler (1) called attention to certain non-virulent organisms closely resembling diphtheria bacilli, and he urged the necessity for differentiation by means of inoculations on guinea pigs, in which animals he had already (1884) described characteristic lesions following the

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inoculation of pure cultures of the diphtheria bacillus. This observation was confirmed in the early nineties by various workers who demonstrated the existence of virulent and non-virulent diphtheria bacilli, as well as pseudo-diphtheria bacilli or diphtheroids.

The claim made by Neisser (2) in 1897, that true diphtheria bacilli could be differentiated from diphtheroids by means of special staining methods, furnished the basis for an immense amount of study of the morphology of the organism. Consequently, the question of virulence was pushed into the background, especially as it was generally assumed that virulence was not necessarily constant for any given strain.

The administrative control of diphtheria resolved itself into a quarantine of the possessors of organisms morphologically resembling diphtheria bacilli, until these organisms were absent from two or more successive cultures. Objection to this procedure, on the ground that such carriers had not been proved to be menaces to the public health, was answered by the statements that avirulent organisms might become virulent, if transferred from one person to another, and that virulence for the guinea pig was not a true index of virulence for man. Furthermore, it was pointed out that the virulence test could never become a routine procedure, because it involved much time and many animals.

However, popular dissatisfaction with the exclusion of carriers from school, solely on morphological evidence, has led to increasing demands for virulence tests with the result that the administrative use of the test is increasing. Confidence in the test has been also much enhanced by experimental evidence of the constancy of virulence of the diphtheria bacillus.

Two changes are necessary before the virulence test can become a part of the routine examination of every morphologically positive diphtheria culture. First, the test must be rendered less expensive of time and animals and, second, the taking of field cultures must be placed on a basis consistent with the epidemiology of diphtheria. These changes would result in a tremendous reduction of the number of cultures to be examined, and would warrant the expenditure of a corresponding amount of laboratory time on the virulence test.

This paper presents the results of virulence tests made by the intracutaneous inoculation of guinea pigs with cultures made from throat swabbings. It is recognized that the so-called virulence test made with cultures of the diphtheria bacillus is not a test of invasive power, but of toxicity. However, custom has apparently sanctioned the former term, and it will be used in the acquired sense in this paper.

(1) *Constancy of virulence of diphtheria bacilli.*

The acceptance of the virulence test as an administrative measure depends on the acceptance of the evidence that there is no tendency for avirulent diphtheria bacilli to become virulent, and that persons harboring these organisms are, therefore, not menaces to the public health. The fact that virulent and avirulent strains could be isolated from the same throat was once considered evidence of change of virulence, but repeated efforts to change the virulence of previously isolated strains have met with failure.

Williams (3) obtained only one variety of virulent organism from the throats of each of ten cases of clinical diphtheria until all suspicious bacilli disappeared. Furthermore, there was no increase in avirulent forms toward the end of the disease, as would have been the case had there been a tendency toward change in virulence. On the other hand, avirulent organisms isolated from healthy throats showed no change after three months of cultivation on media, alone and in symbiosis with virulent streptococci for 90 culture generations, and after repeated animal inoculation. Cary (4) was unable to raise the virulence of avirulent strains by animal passage, either alone or with associated organisms. Meader (5) made subcultures of 25 strains of diphtheria bacilli by means of ten successive platings. Virulence tests were made from the first, fifth and tenth platings with the following result: three strains were constantly virulent, one strain lost its virulence on the third test, two lost their virulence on the second test, and one lost its virulence on the second test, but regained it on the third. On the other hand, two avirulent strains became virulent on the third test, two became virulent on the second test, one became virulent on the second test but lost its virulence on the third test, and 13 strains remained constantly avirulent. That conditions in the throat are not comparable with those in culture media is evidently held by Gelien, Guthrie and Moss (6), who showed that the diphtheria bacilli in the majority of healthy carriers are avirulent. As avirulent bacilli cannot produce diphtheria, and as there is no proof that avirulent organisms acquire virulence, the authors hold that any interference with the liberty of a carrier of diphtheria bacilli, proved to be avirulent for guinea pigs, is unwarranted. Moss, Guthrie and Marshall (7) were unable to change avirulent diphtheria bacilli by experimental inoculation of human subjects. Neither long residence nor repeated transfer increased the virulence of the strain studied. Guthrie, Marshall and Moss (8) inoculated human throats with organisms virulent for guinea pigs, produced clinical diphtheria in those subjects shown to be susceptible by the Schick test, and produced the carrier state in those subjects shown to be immune by the same test. They conclude that virulent diphtheria bacilli retain their characteristics despite long residence in the human throat or transfer from one human being to another. They also conclude that "the guinea pig test is a reliable index of the ability of diphtheria bacilli to cause clinical diphtheria in susceptible human beings." Nuttall and Graham-Smith (9) summarize a review of the literature on constancy of virulence as follows: "Very little evidence can be produced for the statement which is so frequently made, that during prolonged persistence in the throat diphtheria bacilli gradually lose their virulence. Attempts to give virulence to totally non-pathogenic diphtheria bacilli have been almost uniformly unsuccessful, and even attempts to raise the virulence of lowly virulent bacilli have frequently failed. There is also no evidence to show that the virulence of diphtheria bacilli is altered by transference from one individual to another, ..."

Recently, Ladendorff (47) claims to have increased the virulence of clinically avirulent diphtheria bacilli by symbiosis with streptococci. He regards diphtheria bacilli from convalescents and carriers as clinically avirulent, because the subjects from whom the organisms are obtained show no clinical symptoms of diphtheria. He believes, however, that these bacilli may become clinically virulent in case their carrier develops a streptococcus angina. This would account for relapses occurring in diphtheria convalescents. Ladendorff isolated eight strains of diphtheria bacilli from convalescents and carriers, and an equal number of strains of streptococci from various sources. Three guinea pigs were used in each test, the first being inoculated with 0.5 per cent. of its body weight of a broth culture of diphtheria bacilli, the second with a proportional amount of a streptococcus culture, and the third with a mixture of the diphtheria and streptococcus cultures in the quantities used alone, i.e., approximately 1 per cent. of body weight. Seven strains of diphtheria bacilli were virulent for guinea pigs, as were two strains of streptococci. Six of nine animals inoculated with the mixtures died from diphtheria toxemia, from four to nine hours earlier than the control animals inoculated with the corresponding diphtheria strains alone. A strain of diphtheria bacilli which produced loss of weight and oedema when inoculated alone, produced death from diphtheria toxemia 62 hours after inoculation when mixed with an avirulent strain of streptococci. This same strain of diphtheria was inoculated in a mixture with another avirulent streptococcus, and a local streptococcus abscess was produced. On the other hand, a strain of diphtheria bacilli which produced death after 168 hours, when inoculated alone, was mixed with an avirulent streptococcus, and a local streptococcus abscess was the only result.

If Ladendorff is justified in his conclusion that symbiosis with streptococci in the animal body increases the toxin production of diphtheria bacilli, it would appear that an intracutaneous virulence test conducted with field cultures containing diphtheria bacilli and streptococci would be more reliable than a test conducted with pure cultures, for the reason that, under symbiotic conditions, a maximum of toxin production in the animal tissues would be secured.

A review of the literature indicates a preponderance of testimony in favor of the constancy of virulence of the diphtheria bacillus, both in successive cultures isolated from the same person, and in strains carried through many culture generations outside the body. Attempts to change virulence by human inoculation have also failed. The experience of Meader (5) is the only one recently published (except that of Ladendorff) which presents evidence opposed to the general testimony, and it will be noted that 16 of his 25 strains were constant throughout the ten platings.

(2) *Relation of virulence to sources of cultures.*

The relation of the virulence of diphtheria bacilli to the sources of the cultures containing them has been thoroughly studied.

Nuttall and Graham-Smith (10), in the Cambridge epidemic of 1904, found virulent organisms in 48 of 56 persons with clinical symptoms of diphtheria and 38 of 56 persons who had been in definite contact with clinical cases. Pennington (11) examined cultures from the apparently normal throats of 375 school children. Diphtheria bacilli were found in 40 of these children, and pure cultures from 37 were tested for virulence with positive results in five cases. One subject with avirulent bacilli subsequently showed clinical signs of diphtheria, but two subsequent virulence tests were negative.

Pennington also tested diphtheria bacilli isolated from 25 persons, clinically recovered from diphtheria, with positive results in 23 instances. The organisms were isolated either from the last morphologically positive culture taken or from a positive culture obtained a few days before the throat was free from diphtheria bacilli. The cultures were made from 5 to 17 days after clinical recovery. In the two instances where cultures were avirulent, one subject had clinically recovered 110 days, and the other 100 days, before the taking of the culture which was tested. The first of these persons had been discharged on the basis of negative cultures, some time before the virulence test, but showed positive cultures at intervals thereafter. One subject yielded a virulent organism 18 days after clinical recovery and an avirulent organism 17 days later. Typical diphtheria bacilli had not disappeared from this subject's throat 80 days after recovery. Von Sholly (12) secured clultures from 1,000 persons with normal throats, approximately 900 of whom were children under 12 years of age. Diphtheria bacilli were found and tested in 56 instances with 18 positive results. Four of the 18 positive cases were inmates of the nursery of a foundling asylum, and another had been recently exposed to diphtheria. Moss (13) examined cultures from 1,217 Baltimore school children, finding 44 with diphtheria bacilli. Pure cultures isolated from 33 of these carriers were virulent in six instances. Goldberger, Williams and Hachtel (14) made a study of cultures from 4,093 healthy persons, with no history of diphtheria contact, so chosen as to be representative samples of the population of Detroit. Diphtheria bacilli were found in 38 persons. Virulence tests made with pure cultures from 19 of these persons were positive in two instances. These authors also isolated and tested typical organisms from 30 cases of clinical diphtheria with 29 positive results. The single avirulent strain was isolated from a throat culture made during the active stage of the disease. Virulent organisms were, however, isolated from this case over a month later. Weaver (15) isolated diphtheria bacilli in pure culture from 36 clinical cases of diphtheria and 14 carriers. These cultures all gave positive results when tested for virulence by subcutaneous and intracutaneous inoculation of guinea pigs. Blanton and Burhans (16) examined cultures from 8,236 soldiers at Camp Custer, Michigan, and found 148 carriers. Pure cultures were tested from 24 of these carriers with three positive results. During a period of five years Hachtel and Bailey (17) examined 7,831 cultures taken from infected house-holds in Baltimore. Diphtheria bacilli were found in 642 of the cultures. These authors also inoculated 100 pure cultures from diphtheria contacts and found 97 to be virulent for guinea pigs.

Wade and Vaughan (18), during six years, made 1,082 virulence tests by subcutaneous inoculation of guinea pigs, using two strains isolated in pure culture from each case. Their results were as follows:

	Virulent.		Avirulent.	
	No.	Per cent.	No.	Per cent.
Clinical cases	561	85.0	9	2.1
Contact carriers	93	14.1	2	0.5
Non-contact carriers	6	0.9	411	97.4

Of the nine clinical cases harboring avirulent bacilli, six had mild tonsillitis, two had tonsillitis with exudate, and one had had a mild attack of diphtheria 47 days previously. Wadsworth (19) reported 280 positive virulence tests in 306 carriers, convalescent less than three months, and 44 positive tests in 55 contact carriers, of less than three months' duration. He also reported three carriers of virulent bacilli in a group of 32 non-contact carriers in state institutions and schools. Simmons, Wearn and Williams (20) found that diphtheria bacilli isolated from convalescent carriers were virulent in 84.6 per cent. of instances and those isolated from contact carriers were virulent in 48.1 per cent. Beckler, Gillette and Parker (21) made routine examinations of 8,389 cultures from school children in 35 Massachusetts cities and towns. The cultures were taken when diphtheria was present in the school, but the degree of contact was not determined. Forty-one cultures were morphologically positive. Thirty-eight of these were tested for virulence with 36 positive results. The authors consider avirulent diphtheria bacilli to be rare, and believe that many organisms so classed are actually diphtheroids and could be differentiated by close attention to morphology, in cultures not over eight hours old. The results obtained by all the above observers, together with those of several others quoted by Von Sholly, have been summarized in Table I. The percentage of virulent non-contact carriers is computed from a number representing the expectancy of virulent cultures, had all morphologically positive cultures been tested. For example, if 19 of 38 cultures were tested with two positive results, it was assumed that 4 positive results would have been secured, if all 38 cultures had been tested. This method was used by Goldberger, Williams and Hachtel in interpreting the results of the Detroit survey.

TABLE I.

Summary of virulence tests classified according to sources of cultures.

Observers.	Clinical cases and convalescent carriers.		Contact carriers.		Non-contacts.		
	No. pos.	Per cent. virulent.	No. pos.	Per cent. virulent.	No. exam.	Per cent. pos.	Per cent. virulent.
Graham-Smith	56	86	56	68			
Pennington (*a*)					375	10.7	1.3
von Sholly (*b*)					1,000	5.6	1.8
Kober (*a*) (*f*)				8	600	1.6	0.8
Holl trom (*f*)				19			
Aaser (*f*)				20			
Goadley (*f*)				31			
Park and Beebe (*c*) (*f*)				50			
" " " (*d*) (*f*)				10			
" " " (*f*)					330	9.7	2.4
Fibiger (*f*)						11.1	2.9
Moss (*a*)					1,217	3.6	0.7
Goldberger et al.	30	96			4,093	0.9	0.1
Weaver	36	100	14	100			
Blanton and Burhans (*e*)					8,236	1.8	0.2
Hachtel and Bailey			100	97			
Wade and Vaughan	570	98	95	98			
Wadsworth	306	92	55	80			
Simmons et al.		85		48			
Beckler et al. (*a*)					8,389	0.5	0.5
Totals	998	96	320	90	24,240	1.7	0.4 (*g*)

(*a*) school children; (*b*) 900 of group were school children; (*c*) tenement dwellers; (*d*) persons with normal housing conditions; (*e*) soldiers; (*f*) quoted by von Sholly; (*g*) 25.5 per cent. of non-contact carriers harbored virulent bacilli.

The relation of the virulence of diphtheria bacilli to the sources from which the cultures containing them have been derived has been well summarized by Nuttall and Graham-Smith in the following quotations: "The bacilli obtained from patients showing all the symptoms of clinical diphtheria are usually found to possess a high degree of virulence, and it is unnecessary to quote the numerous experiments which have been made. Mention must, however, be made to the fact that attenuated and non-virulent bacilli have on rare occasions been isolated from such cases'' (22). ''The majority of well persons harbouring diphtheria bacilli, which have been derived by contact from clinical cases, retain in their throats fully virulent organisms, and . . . the transference of a virulent diphtheria bacillus from a diseased to an immune person does not tend to weaken its virulence'' (23). ''The proportion of infected contacts is highest amongst the members of the family of the patient, next highest amongst close contacts in hospital wards and institutions, and lowest amongst the more remote contacts in schools'' (24). ''Should the bacilli turn out to be devoid of virulence, and difficulty met with in enforcing isolation, the patient may be discharged with the knowledge that non-virulent diphtheria bacilli have seldom, if ever, been known to give rise to clinical diphtheria or any other lesion, when transferred from one individual to another'' (25).

The same conclusions are expressed by Wade and Vaughan (18): ''We may conclude then that virulent organisms are found almost invariably in persons with clinical symptoms of diphtheria and in well persons intimately associated with them. In well persons who have not associated intimately with infected persons, the organisms, while diphtheria-like, are avirulent.'' Guthrie, Marshall and Moss (8) go a step further in their conclusions as to the relation of the healthy carrier of virulent diphtheria bacilli to the health of the community: ''Although the results of our experiments indicate that the virulent organisms from the throats of healthy carriers are capable of causing clinical diphtheria when the proper opportunity is afforded, we have not obtained evidence, either from this work or from our previous studies of carriers, that diphtheria is spread by their agency under ordinary conditions. It is our belief (1) that such carriers constitute a potential menace to the health of the community, but (2) that their opportunity for dissemination of the disease among their associates is quite limited owing to the relatively small number of persons who are very susceptible to infection, and consequently (3) that the actual part which they play in the spread of diphtheria is probably quite small.''

We have previously stated that the more general administrative use of the virulence test would depend on its simplification and a reduction in the number of routine cultures to be examined for morphological diagnosis of the diphtheria bacillus. The experimental evidence abstracted above shows conclusively that the wholesale culturing of public schools is time-consuming and has many of the inefficient features of the search for the needle in the haystack. We believe that a rational method of diphtheria control should not throw a needless burden on the laboratory, but that the epidemiologist in the field should select his cultures with discretion. Since the clinical cases and their family contacts furnish the sources of infection they should receive painstaking attention. In general, cultures for diagnosis need only be taken from clinical cases and persons definitely exposed to them. Family groups, which include orphanages and boarding schools, are of vastly more importance than public or day schools. Even in family groups the percentage of contact carriers decreases with the improvement of housing conditions. Cultures should be taken from entire day school groups only, when diphtheria prevails, after the identification of all possible clinical cases, convalescent carriers, missed cases and family contact carriers. The burden of field work in diphtheria control falls largely on the public health nurse, and she should receive thorough intruction in the epidemiological principles involved.

Relieved of a large amount of routine diagnostic work, the laboratory could to advantage perform virulence tests on morphologically positive cultures from the following persons: (*a*) convalescents from clinical diphtheria; (*b*) family contacts, as soon as morphologically positive organisms are discovered; (*c*) so-called missed cases; (*d*) persons who have ever had clinical diphtheria or any condition resembling it; (*e*) persons who have a history of a former exposure to clinical diphtheria in the family. Release from supervision might well be conditioned on two negative virulence tests on cultures taken several days apart, without reference to the persistence of organisms morphologically indistinguishable from diphtheria bacilli.

(3) *Relation of virulence to morphology.*

It was anticipated that the improvement of staining methods, initiated by Neisser (2) in 1897, might lead to the differentiation of virulent diphtheria bacilli from the avirulent forms and diphtheroids, on the basis of morphology alone. While this has not been realized, at least some reasonably definite morphological criteria have been formulated which serve as guides to the choice of cultures suitable for virulence tests.

In a study of the virulence of the solid-staining forms of the diphtheria bacillus, Wayson (26) secured only three positive results with 23 cultures. Of these three, two became granular in subcultures from the original field culture containing the solid forms, and remained granular when isolated in pure culture, while the third remained solid-staining. Loeffler's methylene blue was the stain employed. Since young (5 to 15 hours) cultures of virulent organisms may present solid forms and the same is true of cultures taken during convalescence, Wayson concludes: "that the virulence test is at present the most reliable means of determining whether the organism in question will produce diphtheria clinically." Havens (27) regards the solid-staining types as sometimes virulent and, accordingly, considers history of contact as of more importance than morphology in determining the advisability of virulence tests. "If a history of contact with an active case is obtained, the strain should be regarded as virulent until proved otherwise. If there are cases in which no history of such contact can be obtained—usually a small minority of cases—a test for virulence should be made regardless of morphology before such individuals are allowed to become a potential menace to the community. It would seem that morphology as a criterion of virulence has no more foundation in connection with the diphtheria bacillus than does morphology of the pneumococcus, for instance, in ascertaining its virulence." Meader (5) found that cultures containing granular forms were frequently avirulent, as were cultures in which the solid-staining type was constant through many generations. Albert (28) believes that many organisms classified as solid-staining types, when stained with Loeffler's methylene blue, would prove to be either barred or granular, if stained with toluidin blue. No culture which did not contain some granular forms was found to be virulent, when stained by his toluidin blue-iodine method, though some avirulent forms contained granules. Kolmer, Woody and Moshage (29) found the barred and granular types to be virulent in about 70 per cent. of cultures from the throat, nose and ear; the long solid types were virulent in 42 per cent. of cultures; and the short solid type was uniformly avirulent. Calhoun (30) found that certain organisms, classified as diphtheroids by morphology and by fermentation tests, acted as true virulent diphtheria organisms in virulence tests.

The results obtained by these various workers indicate that the morphology of diphtheria bacilli is not absolutely related to the virulence, since typical barred and granular forms may be avirulent. It is evident that any program for the administrative control of diphtheria, based on the disappearance of morphologically positive organisms from cultures, is an injustice to the carrier of avirulent bacilli, and affords no protection to the community from the intermittent disseminator of virulent bacilli. This has been well expressed by Kolmer, Woody and Moshage (29) in the following words: ''Most physicians depend on two or three consecutive negative cultures, before discharging a patient as cured, but when one stops to consider the fallacies of this method, and the goodly proportion of those who escape, still carrying their bacilli, as shown by a more extended series of cultures, it is a question whether or not a good virulence test is not to be preferred. Further than this, we have reason to believe that in some instances the prolonged numbers of positive cultures, following diphtheria, are due to the presence of harmless diphtheria-like bacilli of ''carriers'' that had no connection with the attack of clinical diphtheria. They were present before the attack of diphtheria and are likely to remain for an indefinite time afterward. At first they may be overgrown by the disease-producing bacilli or overlooked in cultures, but as the latter disappear the non-pathogens become prominent again and yield a formidable line of positive cultures.''

(4) *Tests for virulence of diphtheria bacilli.*

According to Nuttall and Graham-Smith (31) ''a correct estimate may be formed of the virulence of the bacilli toward guinea pigs, and satisfactory evidence is yet lacking to show that races, non-virulent to guinea pigs, can produce serious disease in man. The results of those experiments which have been made under uniform conditions, i.e., the inoculation of well-grown 48-hour cultures, in sugar-free broth or ascitic broth, into half-grown guinea pigs, show that the very great majority of races of diphtheria bacilli are either fully virulent or totally non-virulent for these animals.''

(*a*) *Subcutaneous virulence tests.* The subcutaneous virulence test consists in the inoculation of a guinea pig with a culture containing diphtheria bacilli and, if death occurs, examining the animal for local hemorrhagic or gelatinous oedema and congestion of the suprarenal glands, varying from pink to dark red. The cultures used vary from saline suspensions of the 24-hour growth on Loeffler's blood serum slants to 72-hour cultures in dextrose broth. A 24-hour culture in ascitic broth has given excellent results. According to

Kolmer and Moshage (32) no material possesses any advantages over the saline suspension from Loeffler's slants. This would seem to be a most reasonable conclusion, when it is considered that the test is being made of the toxicity of the organisms for animals, rather than their toxin production in broth. The amount of culture injected may vary with the weight of the animal, or may be a fixed dose for animals within a certain range of weight, e.g., 4 c.c. of the growth on a Loeffler's slant, suspended in 10 c.c. of physiological salt solution, for guinea pigs weighing from 250 to 300 gms. (Kolmer and Moshage). In some instances the decision as to virulence is based on the necropsy findings, no control being used. When control animals are used they are inoculated either with a mixture of antitoxin and the material to be tested, or are protected by antitoxin given before the material is inoculated. The interval between the administration of antitoxin and the test may vary from 24 hours to a few moments.

The most radical departure from the above methods has been suggested by Wayson (26), who compared virulence tests with field and pure cultures of the same organisms. "This point," writes Wayson, "was suggested by the practice in some diagnostic laboratories of injecting into a guinea pig, as a test for virulence, 24-hour mixed cultures washed from a slant, without using a control." The material inoculated by Wayson consisted of 0.1 to 1.0 c.c. of the growth on a Loeffler's slant, suspended in 5 c.c. of salt solution. The slants had been inoculated 24 hours previously from throat swabbings, and virulence tests were made as soon as possible after the morphological diagnosis. Organisms from these field cultures were also isolated in pure culture and tested. Antitoxin-protected controls were used in all cases. Of 25 cultures tested in this manner, only one failed to show agreement between the pure culture and the corresponding field culture. In this instance, the animal inoculated with the pure culture died, the one inoculated with the field culture survived. Wayson advocates the method as reliable and also time-saving, when compared with the methods involving the subcutaneous inoculation of pure cultures.

(*b*) *Intracutaneous virulence tests.* In 1909 Roemer (33) described a reaction, produced by the intracutaneous inoculation of diphtheria toxin, and suggested a method for the standardization of toxin and antitoxin using this reaction as a basis. With very small doses of toxin, the reaction consists of a slight oedematous swelling and redness for three or four days, which is followed by a falling of the hair over the inoculated region. With medium doses, a little necrosis is apparent, beginning the first day as an infiltration on the inoculation site. With larger doses, corresponding to about 1/50 of the subcutaneous M.L.D., a marked superficial necrosis appears on the third or even the second day. The reaction is produced by the injection of 1/500 to 1/250 M.L.D. Glenny and Allen (34), who have employed Roemer's intracutaneous method of standardizing toxin and antitoxin with satisfactory results, state that 1/500 M.L.D. of diphtheria toxin inoculated intracutaneously into a previously depilated guinea pig produces a well-defined red area about 15 mm. in diameter at the end of 36 hours. After the third day the color fades, leaving a slightly discolored bare patch contrasted with the normal skin, where hair has begun to grow. Where the amount of toxin is excessive, a scab is formed after four or five days. Novotny and Schick (35) secured a diphtheria toxin which had been concentrated to ten times normal strength, and rubbed it into circular abrasions made through the epidermis of guinea pigs, according to the method of Pirquet. Within 24 hours there developed at the inoculation site a 5-mm. red papule, surrounded by an areola 12 mm. in diameter, which increased to 25 mm. on the second day. On the third day, the central infiltration had become a 10-mm. whitish "blister," as if from a burn, surrounded by a markedly infiltrated area, with a diameter of 20 mm. During the fourth day the "blister" dried to a brownish crust which finally became detached, leaving a rounded ulcer with an uneven base and sharply indented borders which gradually healed by granulation. The authors further showed that 200 units of antitoxin, administered five hours after the inoculation of the toxin, exerted a marked influence on the reaction, this influence being much greater when the antitoxin was administered intravenously. A series of guinea pigs inoculated with toxin at intervals after the administration of 200 units of antitoxin showed partial reactions when the inoculation was made eight days after passive immunization, advanced reactions when inoculated 14 days after immunization, and complete reaction, i.e., total loss of immunity, when inoculated 20 days after immunization.

Neisser (36) employed living cultures, instead of toxin, and found that the results from intracutaneous inoculation of 23 pure cultures of diphtheria bacilli paralleled those from the subcutaneous inoculation of the same cultures. In no instance was there a positive intracutaneous result where the subcutaneous test was negative, although the intracutaneous test, according to Roemer, is positive with amounts of toxin as small as 1/500 M.L.D. This is a further confirmation of the statement already quoted to the effect that strains are either avirulent or fully virulent, when tests are made under uniform conditions (Nuttall and Graham-Smith). Neisser suspended a loopful of the growth on a 24-hour Loeffler's slant in 10 c.c., 100 c.c. and 1,000 c.c. of salt solution, and injected 0.1 c.c. of each suspension intracutaneously into depilated spots on a guinea pig. A fourth depilated spot received 0.05 c.c. of the first suspension, mixed with an equal quantity of antitoxin containing about one half a unit. Neisser warns against the possibility of producing a general immunization, through excess of antitoxin, which would inhibit the effect of the toxin at the three other inoculated spots. He describes the positive results as an infiltration, inflammation and later a necrosis.

Zingher and Soletsky (37) modified the technique of Neisser in the following particulars: (*a*) Instead of using a mixture of the culture and antitoxin on the same animal, with three dilutions of the culture, a control animal was given 250 units of antitoxin, intraperitoneally 24 hours before the test, or intracardially at the time of the test. (*b*) Instead of using three dilutions of the culture on one animal, the entire surface of the Loeffler's slant was washed with 20 c.c. of salt solution and 0.15 c.c. of the washings were inoculated into depilated spots on the normal animal and its control. Four to six different cultures could thus be tested on two animals which resulted in a decided saving of animals. The authors described the positive reaction as a definitely circumscribed inflammatory lesion which showed a superficial necrosis in from 48 to 72 hours. No necrosis is observed with avirulent bacilli. The skin of the protected animal remained normal at the inoculation sites of both virulent and avirulent strains. Zingher (38) in a later publication describes the lesions due to the injection of toxin in terms of redness; slight, medium or marked induration; scaling and slight or marked necrosis. He advocates pulling out the animal's hair in preference to the use of a depilatory or shaving, both of which may interfere with the interpretation of the reaction through irritation. Smeeton (39) isolated 37 pure cultures of diphtheria bacilli from the throats of known or suspected clinical cases and compared their virulence by the intracutaneous method of Zingher with that by subcutaneous inoculation of 48-hour serum broth cultures. Control animals were used in all tests. Twenty of the 37 cultures gave identical results by both methods. On account of the loss of several animals by an infectious disease, the remaining 15 tests had to be repeated, but finally the results corresponded. Smeeton noted that the suprarenal bodies of the animals dead from the effects of diphtheria toxin showed varying degrees of congestion. In one instance the diphtheria bacillus was isolated from the heart's blood, but the suprarenal bodies were apparently normal. Smeeton concludes that the intracutaneous method is more accurate than the subcutaneous method, as well as being more saving in time and animals.

Among the requisites for a reliable virulence test, Kolmer and Moshage (32) insist on (*a*) delicacy, in order to appreciate degrees of virulence; (*b*) representative sampling, i.e., the test should be made with a mixture of bacilli from several colonies of the culture, in order to avoid danger of picking only one strain, when virulent and avirulent strains coexist in the same individual; and (*c*) specificity, i.e., the test should be made with pure cultures since "cultures contaminated with other micro-organisms may show no evidence of virulence because of alteration of the culture medium or the presence of products of the metabolic activity of the contaminating organism." Kolmer and Moshage secured 86.5 per cent. of positive results by the subcutaneous inoculation of 72-hour plain dextrose broth cultures of diphtheria bacilli, and 64.9 per cent. of positive results by the intracutaneous inoculation of the same cultures. After a comparison of cultures, grown in various ways, they concluded that the subcutaneous inoculation of the saline suspension from a 24-hour Loeffler's slant is the preferred method. It is to be regretted that no intracutaneous tests were made with the saline suspension for comparison with the results with dextrose broth cultures. Wayson (26) made four sets of intracutaneous tests, using four tests to one guinea pig, with saline suspensions from 24-hour cultures on Loeffler's slants. The slants were washed with 20 c.c. of saline and 0.2 c.c. were inoculated. He reported that redness and infiltration of the skin were produced in several instances by avirulent pure cultures, but does not mention superficial necrosis. "In one instance a culture, producing a slight skin reaction, killed on subcutaneous injection, while the antitoxin control survived." Wayson does not mention how many of his 16 tests were made with pure cultures, and how many with field cultures. That some field cultures were used, however, is apparent since he writes: "The intradermic reactions, with the use of mixed cultures, are complicated by pyogenic organisms, ulceration occurring frequently.'' Unfortunately, Wayson gives no description of the intracutaneous lesions observed, and no estimation of the effect of the contaminating organisms on the intracutaneous reaction can be made. The impression conveyed is that the subject of intracutaneous virulence tests with field cultures had been touched upon rather than investigated. This impression was confirmed in a personal interview with Dr. Wayson who advised one of us (J. N. F.) to reopen the subject, stating that no records of his work were available, and that his tests had been too few in number to warrant definite conclusions.

Weaver (15) found no discrepancy between the subcutaneous and intracutaneous reactions of 26 pure cultures of diphtheria bacilli. Calhoun (30) reported satisfactory results with the method of Zingher and Soletsky, when testing six or eight cultures on one guinea pig. Eagleton and Baxter (40) modified the technique of Zingher and Soletsky by (*a*) standardizing the saline suspension to 50,000,000 bacilli to the c.c. by opacity; (*b*) using a strain known to be virulent in the group tested on each animal; (*c*) administering a dose of 500 units of antitoxin to the control animal on the day preceding the test; and (*d*) giving a following dose of 125 units of antitoxin to the normal animal four or five hours after the tests had been put on. The authors claim that the last-mentioned procedure does not affect the local reaction, but protects the animal from death when several virulent cultures have been inoculated. They define a positive reaction as a rose-red swelling which may terminate in slight necrosis. Contamination of the test culture by streptococci may obscure the result by causing a reaction in both the normal and protected animals, though this is not constant. In making virulence tests Rickards (41) noted that colonies from the same original cultures varied in virulence. This coexistence of virulent and avirulent organisms has been commented upon by Wade and Vaughan, Nuttall and Graham-Smith, Kolmer and Moshage and others. This possibility adds to the difficulty of making tests with pure cultures since, after the field cultures are plated, several colonies must be picked and either isolated or pooled. The field culture, as employed by Wayson, contains all the organisms from the original source which are capable of growth on Loeffler's blood serum. Furthermore, the transfer from human tissue to animal tissue is made within 24 hours, thus lessening the possibility of changes in the organisms through repeated cultural transfer. There is, however, no saving of animals possible in any modification of the subcutaneous test. It was with the hope of effecting a saving, while preserving the rapid features of Wayson's method, that the following observations were made on the results of intracutaneous inoculations of field cultures of diphtheria bacilli.

II. TECHNIQUE OF INTRACUTANEOUS VIRULENCE TESTS WITH FIELD CULTURES.

Practically all of the cultures tested by us were obtained from the State Hygienic Laboratory. They were on Loeffler's blood serum slants which had been inoculated on the day preceding the virulence test, from throat swabbings received by mail. The cultures were removed from the incubator at 9:00 A.M., after from 17 to 24 hours' incubation, for the purpose of making stained smears for diagnosis. Cultures yielding positive smears were turned over to us and kept at room temperature until the afternoon when virulence tests were made. The smears for diagnosis were stained with Ponder's acetic toluidin blue, and all cultures considered morphologically positive by us showed barred or granular forms. After preparing a check smear from the culture, 2.5 c.c. of physiological salt solution were added to the slant and the growth scraped from the surface by means of a platinum loop. The suspension was then transferred to a sterile test tube, placed in a comparator block and diluted with salt solution to the same turbidity as that of a standard tube. The standard turbidity tube was prepared as follows: A test tube (19 by 150 mm.) containing 10 c.c. of plain melted agar was allowed to solidify on a 10 per cent. slope. The surface was completely inoculated with typhoid bacilli, and the tube incubated for 24 hours, after which the growth was washed off with 10 c.c. of salt solution, transferred to one of the small test tubes, which are used with the comparator block, and sealed. After standardization of the suspension from the Loeffler's slant, it was transferred to a sterile Petri dish, from which it could easily be drawn into syringes as required. The long hairs were plucked from the sides of a white guinea pig and the residual short hairs shaved. Enough of the standardized suspension of each culture was injected intracutaneously to produce a bleb approximately 10 mm. in diameter (0.15 to 0.2 c.c.). Two cultures were inoculated on each side of the animal. Ordinary glass tuberculin syringes were used, with 26-gauge needles, having a very short bevel. Control inoculations were made on a guinea pig which had received 500 units of antitoxin a few moments previously. Subcutaneous inoculations of one c.c. of the same culture suspension were made in a normal guinea pig and in one protected by 500 units of antitoxin administered subcutaneously a few moments previously. In view of the findings of Novotny and Schick, heretofore noted, we saw no advantage in administering the antitoxin on the day preceding the test, especially as we were unable to anticipate the number of positive cultures which would be available for testing each day. The inoculated animals were observed daily for five days and measurements were made of all lesions. Animals dying were examined for the usual signs of the action of diphtheria toxin, and if these were not found, cultures were made from the heart's blood.

III. Appearance of intracutaneous lesions.

Before beginning the discussion of the results of our series of intracutaneous tests with field cultures, a description will be given of lesions produced by (1) diphtheria toxin in varying dilutions; (2) pure cultures of diphtheria bacilli, both virulent and avirulent; (3) field cultures of virulent and avirulent diphtheria derived from throat swabbings; (4) filtrates of suspensions of field cultures; (5) a virulent culture inoculated into an animal previously injected with horse serum; (6) a virulent pure culture three months after isolation; (7) field cultures derived from throat swabbings containing no diphtheria bacilli.

1. *Lesions produced by diphtheria toxin in varying dilutions.* (*a*) A normal guinea pig was inoculated intracutaneously in two spots on the right side with 0.2 c.c. (1/40 M.L.D.) of Schick toxin, and in two spots on the left side with 0.2 c.c. of Schick control, both supplied by the New York City Health Department. A guinea pig protected by the subcutaneous inoculation of 500 units of antitoxin was similarly inoculated. Within 24 hours there appeared at the sites of the two toxin inoculations in the normal animal circular areas of redness which had attained a diameter of 18 mm. at the end of 48 hours. By the fourth day, the redness had faded to a light brown or tan color, and on the fifth day the epidermis within the areas showed superficial scaling. The heated toxin control, in this animal, and both toxin and heated control in the protected animal produced no lesions other than the slight needle trauma. (*b*) A

normal guinea pig was inoculated with 0.1 c.c. of Schick toxin (1/50 M.L.D.) and 0.1 c.c. of Schick control both supplied by the Cutter Laboratory. A protected animal was similarly inoculated. Within 24 hours there appeared at the toxin inoculation site a slightly elevated reddened area which measured 15 mm. on the second day, faded to brownish tan on the third day, and began scaling on the fourth day. There were no reactions from the heated control or on the protected animal. (*c*) A normal guinea pig was inoculated with 0.1 c.c. of diluted toxin containing one M.L.D., a second animal received 0.5 M.L.D. and a third, 0.1 M.L.D. Within 24 hours a slightly elevated reddened area about 20 mm. in diameter appeared at each inoculation site. The redness and induration reached its maximum on the second day, measuring from 25 to 30 mm. in diameter. Within this red area there appeared, on the second day, a white area which measured from 15 to 20 mm. As the white area increased its center became purple, measuring 10 to 15 mm. on the second day. On the third day the redness and induration had slightly decreased, the white area was still present in the lesions produced by the 0.5 M.L.D. and 0.1 M.L.D. doses of toxin, but had been entirely replaced by an extension of the central purple area in the lesion produced by one M.L.D. In all these lesions the purple center had become darker and flakes of epithelium had become detached. On the fourth day the redness and induration had decreased in diameter and amount, and the necrotic area had changed from a blackish purple to a dark brown crust. The guinea pig which had received one M.L.D. of toxin died on the fourth day and showed the internal effects of diphtheria toxin on examination. (*d*) A normal guinea pig received 0.1 c.c. of diphtheria toxin containing 10 M.L.D. and the same dose of a heated control. A second guinea pig, protected by 1,000 units of antitoxin, was similarly inoculated. The normal animal died from diphtheria toxemia within 48 hours with no local result from the intracutaneous inoculation other than a purple area measuring 5 mm. in diameter. The protected animal showed a transient redness on the site of the inoculation of heated toxin and a reaction comparable to those described in (*c*) on the site of inoculation of the 10 M.L.D. of toxin.

The reaction produced by the intracutaneous inoculation of Schick toxin (1/50 to 1/40 M.L.D.) in guinea pigs follows practically the same course as in man, i.e., redness and slight induration followed by browning and superficial scaling. With amounts of toxin ranging from 0.1 to one M.L.D., each of the above stages in the reaction is intensified, i.e., the redness and induration are marked, the browning and superficial scaling is replaced by a dead white area which turns almost black, while large pieces of epithelium separate, exposing the derma. The most characteristic appearance of the reaction is at the ''target'' stage, in which are seen concentric areas of purple, white and red. When the crust finally forms and contraction takes place, the edges of the healing lesions may become elevated as noted by Novotny and Schick. At the height of the reaction, however, one is impressed by the flatness and centrifugal tendency of the necrosis, as distinguished from the elevation and centripetal tendency of a local streptococcus infection.

2. *Lesions produced by pure cultures of diphtheria bacilli.* Normal and protected guinea pigs were inoculated with three pure cultures of diphtheria bacilli known to be virulent and two pure cultures known to be avirulent. The lesions produced by the virulent bacilli closely paralleled those produced by 0.1 M.L.D. of toxin, but were somewhat less intense. Redness and induration appeared on the first day, central darkening on the second, sloughing of the epidermis measuring about 10 mm. in diameter on the third day, and a crust formed on the fourth or fifth day. The protected animals showed slight induration and redness during the first 24 hours which practically disappeared on the third day. The lesions produced by the avirulent diphtheria bacilli were of the same transitory type.

3. *Lesions produced by field cultures of diphtheria bacilli derived from throat swabbings.* Normal and protected guinea pigs were inoculated with three field cultures containing diphtheria bacilli known to be virulent and two field cultures containing avirulent organisms. The three virulent field cultures produced reactions which paralleled those produced by pure cultures, i.e., redness and induration followed by a necrosis which manifested itself by a white area with an extending purple center, terminating in sloughing of the epidermis and crust formation. In one reaction the purple center had already appeared at the end of 24 hours, another showed the white area at this time, while the third reaction showed only the usual redness and induration. The protected animals inoculated with the virulent field cultures showed marked redness and induration of less diameter than in the normal animals. In some instances a central brown crust appeared at the end of 48 hours, after which the redness and induration rapidly disappeared. This brown crust in no way resembled

the white and purple concentric area above described, and could not be confused with it unless the lesions were not observed before the third day. Even when this brownish crust was surrounded by a yellowish white area, due to the formation of pus by the organisms contaminating the field culture, there was little danger of confusing the lesion with the ''target'' lesion above described. In the case of a combined positive, due to diphtheria bacilli in conjunction with a contaminating pus-producing organism, the inflammatory center is surrounded by a marginal zone which is characterized by a dead white color, later replaced by purple, accompanied by sloughing of the epithelium. This marginal zone is, of course, absent from the lesion on the protected control animal, and any lesions where sloughing occurs on both animals are not produced by diphtheria toxin. In this latter, most unusual event, the test should be repeated as there is a possibility of masking a positive reaction on the normal animal. The two avirulent cultures produced similar results in the normal and protected animals. The characteristic white area with the purple center was not produced.

4. *Lesions produced by filtrates of suspensions of field cultures.* These observations were made at the suggestion of Professor Ivan Hall in order to determine, if the toxin responsible for the intracutaneous reaction was present in the bacterial suspension before inoculation. Were this the case, the test could be made with bacteria-free filtrates containing toxin. Standard suspensions were accordingly made of the 24-hour growth on Loeffler's slants inoculated from four throat swabbings. The suspensions were placed in Berkefeld filters and suction applied until about half of the fluid had passed through. The right sides of four guinea pigs were inoculated intracutaneously with the unfiltered suspension and the left side with the filtrate. Beyond a slight transient redness in two instances, the left sides remained normal, while the right sides showed typical lesions. It would appear, therefore, that the toxin production on solid media is not sufficient to produce a visible reaction. On the other hand, apparently nothing would be gained by growing the organism on a medium favorable to toxin production, since the reaction is evidently caused by toxin produced by the organism after inoculation. Possibly this furnishes an explanation for the observation made by Kolmer and others that virulence tests made with suspensions of organisms grown on solid media were as satisfactory as those made from broth cultures.

5. *Lesions produced by a virulent culture inoculated into an animal previously injected with horse serum.* It was found that normal horse serum exerted no protective influence against a virulent culture.

6. *Lesions produced by a virulent pure culture, three months after isolation.* Characteristic necrosis was produced by a virulent culture three months after isolation.

7. *Lesions produced by field cultures containing no diphtheria bacilli.* It was found that nothing resembling necrosis was produced by the intracutaneous inoculation of field cultures containing no organisms morphologically resembling *B. diphtheriae.*

From a study of the lesions described, it appears that the intracutaneous effect of diphtheria toxin is fairly constant, whether injected as such or developed after the inoculation of pure or field cultures of diphtheria bacilli. The constant feature of these lesions is a necrosis, varying degrees of which have been described. The next step is obviously the application of the intracutaneous virulence test to a large number of field cultures derived from throat swabbings.

IV. Virulence tests with field cultures.

Two hundred and forty-seven cultures made on Loeffler's medium from throat swabbings were tested by the intracutaneous method. Two hundred and two of these cultures were also tested by the subcutaneous method, advocated by Wayson. All tests were controlled by antitoxin-protected animals. The cultures all contained either barred or granular organisms, morphologically indistinguishable from *B. diphtheriae.*

These cultures were derived from the following sources:

1. Unknown. Swabbings had been received without information as to condition of subject from whom obtained.

2. Tonsillitis. These cultures were from persons with simple sore throat as well as tonsillitis.

3. Diphtheria with clinical symptoms, for diagnosis. These cultures were from persons showing definite clinical symptoms of diphtheria. They had been received at the State Hygienic Laboratory for diagnosis.

4. Diphtheria, convalescent, for release. These cultures were taken from convalescent diphtheria patients to serve as a basis for release from quarantine on two negative reports.

5. Contact, definite. These cultures were from persons with a definite history of exposure to a patient with diphtheria. They were received either for diagnosis of the carrier state or for release where the carrier state had been previously determined.

6. Contact, indefinite. In this group were cultures from persons who had no history of contact with a patient with diphtheria, irrespective of contact with a carrier. The cultures were either received for diagnosis as part of a group survey, e.g., of a school, in order to determine the existence of carriers, or were received for release where the carrier state had been previously determined.

Organisms producing the previously described toxin necrosis when inoculated intracutaneously were considered to be virulent diphtheria bacilli.

This typical necrosis appeared on the intracutaneously inoculated animal four days before the death of the subcutaneously inoculated animal in one instance, three days in one instance, two days in 10 instances, and one day in 21 instances. The necrosis appeared on the intracutaneously inoculated animal on the day of death of the subcutaneously inoculated control in 50 instances, and on the day after the death in 10 instances. It follows, therefore, that approximately one third of the virulent cultures were diagnosed by the intracutaneous test one or more days before the same cultures were diagnosed by the subcutaneous method.

Some measurements of the necrotic areas produced by the virulent cultures are presented. The measurements were taken of the actual area of necrosis without reference to the surrounding area of redness and induration. Apparently, in the great majority of cases, the necrotic area exceeded 10 mm. in diameter, which, it will be recalled, is the size of the bleb produced by the intracutaneous injection of the saline suspension prepared from the culture.

Diameters in mm.	Number measured each day following inoculation.			
	II.	III.	IV.	V.
5 to 9	4	8	2	
10 to 15	16	31	19	6
16 to 24	7	19	16	8
25 and over		3	9	6

The results of these tests are summarized in Table II. It will be noted that the appearance of necrosis in the animal inoculated intracutaneously was confirmed by the appearance of typical diphtheria toxin lesions at autopsy, following the death of the animal inoculated subcutaneously, except in the following six instances: In four tests the normal guinea pigs which had been inoculated subcutaneously survived, while the animals inoculated intracutaneously with the same cultures showed definite necrosis. In two instances the normal animals inoculated subcutaneously died of streptococcus infection while the animals inoculated intracutaneously showed definite necrosis. In 11 tests the normal guinea pigs which had been inoculated subcutaneously died either from streptococcus infection or some cause other than diphtheria. The normal guinea pigs inoculated intracutaneously from the same cultures showed no signs of necrosis.

From the above experiences it appears that the subcutaneous virulence test, made with field cultures, is open to greater confusion than the intracutaneous test with field cultures. In 13 of the above tests the death of subcutaneously inoculated animals was due to some other cause than diphtheria toxin. On the other hand, four subcutaneously inoculated animals survived though inoculated with cultures capable of producing characteristic diphtheria toxin necrosis in intracutaneously inoculated animals.

The preponderance of virulent diphtheria bacilli in clinical cases and convalescents, as compared with those occurring in contacts, is in accordance with the results obtained by the observers listed in Table I. The absence of any carrier of virulent organisms from the indefinite contact group may be explained by the rigid exclusion from this group of any person who had a history of definite contact with a diphtheria patient. The histories in all doubtful cases were verified by correspondence with health officers and public health nurses in the communities from whence the cultures were received.

Fifteen pure cultures were isolated from the corresponding field cultures and were tested for virulence by the intracutaneous method. Six were tested once, five twice, three three times, and one four times. No discrepancy was noted between the virulence of the pure culture and the field culture from whence it was isolated, and no change in virulence was noted on repeated inoculation.

Twenty-nine of the cultures listed in Table II were second and three were third cultures from the same persons. In three instances the virulence of the second culture did not agree with that of the first culture from the same person. Two of these persons were convalescents whose virulent organisms were apparently supplanted by avirulent organisms. The third person was a patient with clinical symptoms of diphtheria, whose first culture contained avirulent organisms and whose second culture, tested during convalescence, contained virulent organisms. Details of the tests made with cultures from these three subjects will be found in Table III which also gives details of negative virulence tests with cultures from four persons with clinical diphtheria. It must be borne in mind that barred or granular diphtheria organisms were present in all of these cultures. The clinical histories obtained from the physicians leave little doubt as to the correctness of the diagnosis. There was no discrepancy between the subcutaneous and intracutaneous methods in any of these four tests.

As has been explained, the cultures listed in Table II were all obtained from the State Hygienic Laboratory which at that time was recommending release from quarantine on two morphologically negative cultures. In a number of instances we were able to determine the number of days from diagnosis to release, and this information is presented herewith for comparison with the time between diagnosis and determination of virulence.

Culture No.	Days from diagnosis to release on two negative cultures.	Days from diagnosis to determination of virulence.	
		Positive.	Negative.
22	53		47
25	23		18
29	52		48
46	36		30
47	26		26
13(30)	299		274(288)
58(71)	46		29(31)
36	31		21
31	127	116	
26	31	13	
50(68)	16	4(12)	
77	43	31	
80	59	51	
84	24	19	
94	9	5	

In the case of the eight persons harboring avirulent organisms it

TABLE II.

Virulence tests with field cultures containing organisms morphologically indistinguishable from barred and granular diphtheria bacilli.

Result of test.		Source of culture and diagnosis of virulence.															
Intracutaneous animal.	Subcutaneous animal.	Unknown.		Tonsillitis.		Diphtheria.				Contact.							
										Definite				Indefinite			
						Clinical cases for diagnosis.		Con-valescents for release.		for diagnosis.		for release.		for diagnosis.		for release.	
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Necrosis.......	Death from diphtheria toxin	4	....	8	....	41	....	27	....	7	....	5					
Necrosis.......	Death from streptococcus infection...............	....	....	....	....	2											
Necrosis.......	Survival.................	....	....	....	....	....	....	2	....	2							
No necrosis....	Death from streptococcus infection...............	....	1	....	3	....	....	....	2	....	1	....	1	....	....	....	2
No necrosis....	Death from other cause than diph. or strep...........	....	....	....	....	....	....	....	....	....	1						
No necrosis....	Survival.................	....	2	....	28	....	5	....	17	....	10	....	11	....	3	....	17
Necrosis.......	None used..............	....	....	....	....	2	....	....	....	5	....	3					
No necrosis....	None used..............	....	....	....	1	....	....	....	....	....	5	....	2	....	26	....	1
Totals.......	....	4	3	8	32	45	5	29	19	14	17	8	14	0	29	0	20

TABLE III.

Virulence tests whose results did not agree with those anticipated from the source of the culture or whose results were not constant when repeated with a second culture.

No. of culture.	Source of culture.	Result of subcutaneous inoculation.		Lesions resulting from intracutaneous inoculation.		Diagnosis.
		Normal animal.	Protected animal.	Normal animal.	Protected animal.	
24....	Clinical diphtheria for diagnosis.	Survival.	Survival.	Maximum 15 mm. diameter on 3d day, red and indurated.	Max. 10 mm. on 1st day, red and indurated; decreased.	Neg.
26....	Clinical diphtheria for release.	Death diph. toxin 1st day.	"	44 mm. on 3d day, red and indurated; necrosis 20 mm.	21 mm. on 1st day, red and indurated; decreased rapidly.	Pos.
36....	Same as 26, 9 days later.	Survival.	"	13 mm. on 1st day, red and indurated; decreased rapidly.	15 mm. on 1st day, red and indurated; decreased rapidly.	Neg.
90....	Clinical diphtheria for diagnosis.	"	"	20 mm. on 2d day, slightly red and slightly indurated.	11 mm. on 1st day, red and indurated.	Neg.
106....	Clinical diphtheria for diagnosis.	Death diph. toxin 2d day.	"	29 mm. on 2d day, red and indurated; necrosis 14 mm.	11 mm. on 2d day, red and indurated.	Pos.
146....	Same as 106, 60 days later.	Survival.	"	12 mm. on 1st day, red and indurated; decreased slowly.	7 mm. on 1st day, red and indurated; decreased rapidly.	Neg.
170....	Clinical diphtheria for diagnosis.	"	"	12 mm. on 2d day, red and indurated; decreased slowly.	4 mm. on 1st day, slightly red and indurated.	Neg.
171....	Clinical diphtheria for diagnosis.	"	"	14 mm. on 2d day, red and indurated; decreased slowly.	2 mm. on 2d day, slightly indurated; slightly red.	Neg.
180....	Same as 171, 6 days later.	Death diph. toxin 4th day.	"	30 mm. on 3d day, red and indurated; necrosis 7 mm.	5 mm. on 1st day, sl. red; sl. indurated.	Pos.
201....	Clinical diphtheria for diagnosis.	Survival.	"	15 mm. on 1st day, red and indurated; decreased rapidly.	5 mm. on 1st day, sl. red; sl. indurated.	Neg.

is evident that much time might have been saved by release on the basis of a virulence test, rather than on two negative cultures. In the case of the seven persons harboring virulent organisms, there is a disquieting suggestion that release on two morphologically negative cultures might have been premature and that release on two negative virulence tests would have been more conservative of the public health.

V. SOURCES OF FAILURE IN VIRULENCE TESTS WITH FIELD CULTURES.

Eighteen cultures tested were not included in Table II for the following reasons: Eight did not contain granular or barred forms and produced neither necrosis, when inoculated intracutaneously, nor death from diphtheria toxin when inoculated subcutaneously. One culture showed positive virulence by both methods, but no history of the case was obtainable other than that the culture was from a carrier for release. The remaining nine cultures exemplify possible sources of failure in ability to interpret the reaction produced.

1. Accidental death. Three cultures were tested on an animal which met with accidental death within 24 hours after inoculation.

2. Several positive cultures on one animal. Four cultures were on one animal which died on the second day with evidence of diphtheria toxemia at autopsy. The skin lesions were not far enough advanced to determine which of the four cultures was responsible for death. A later communication will deal with the possibility of avoiding this result by the use of a following injection of antitoxin, as suggested by Eagleton and Baxter (40).

3. Failure to produce necrosis. One culture produced redness and induration with a central yellowing, but no definite necrosis. The subcutaneously inoculated control animal died with signs of diphtheria toxemia. As against this, however, we had four cultures which produced definite necrosis in the intracutaneously inoculated animals, but did not affect the subcutaneously inoculated controls.

4. Death due to streptococcus infection. One culture produced an erysipelas-like inflammation with death of the animal on the second day. This inflammation completely obscured the skin reaction. The subcutaneously inoculated animals, both normal and antitoxin-protected, died of the infection. The diphtheria organisms isolated from the culture containing the streptococcus were found to be avirulent. On the other hand, we had 12 cultures which caused death of the subcutaneously inoculated animal from streptococcus

infection, but showed no necrosis on the animal inoculated intracutaneously. It would appear that the chance for failure introduced by the streptococcus in field cultures is comparatively small and, in actual practice, would necessitate the repetition of very few tests.

VI. Advantages of virulence tests with field cultures.

Even should it be necessary to isolate an organism in pure culture occasionally, it is evident that the test with field cultures is saving both in animals and time. With a minimum of four tests to one animal and its protected control, we have a rate of two tests to one animal, as contrasted with two animals to one test, which is the rate for the subcutaneous test. This represents a saving of three animals for each two tests. The most decided advantage of the test, however, is in the saving of laboratory time. The morphological diagnosis can be made in the morning, the virulence test made in the afternoon, and the result obtained usually on the second day following the discovery of a morphologically positive culture. In fact, instead of reporting the result of a carrier survey of a school in terms of morphology, it is possible to report in terms of virulence of bacilli, which obviates the necessity of exclusion and readmission of the majority of the carrier group.

VII. The application of the virulence test to the administrative control of diphtheria.

Probably the most unpopular public health procedure, from the standpoint of the laity, is the restriction of the liberty of diphtheria carriers. It has been the cause of numerous legal proceedings to say nothing of constant wrangling between health officers and the public. There are many instances on record where a re-examination of cultures from a carrier released on two negative cultures has shown the presence of diphtheria bacilli. At best, release on two negative cultures is a makeshift procedure, entered into with the hope that the disappearance of organisms is final. The restrictions imposed on carriers do not affect adults as much as school children. With the latter there is loss of school time, costly alike to the child and to the state.

The carrier state appears to be capable of indefinite continuance, in spite of antiseptic sprays, vaccines and tonsillectomies. The virulence of the organism has little to do with the duration of the carrier state. There is no evidence that the obstinate carrier is harboring virulent organisms and, since this is the case, the virulence test becomes a matter of simple justice due him. It is manifestly impossible to accompany every morphological diagnosis with a virulence test. It is manifestly unjust to restrict carriers of avirulent organisms. There must be a working rule which shall conserve the rights of the individual as well as protect the public health. At present the tendency seems to be to wait an arbitrary time for the bacilli to remain absent from two cultures, at least 24 hours apart. Virulence tests are performed on diphtheria bacilli persisting beyond this time.

Ruh, Miller and Perkins (42) would define a true carrier as a person who harbors virulent diphtheria bacilli for a period of 21 days or more. "While this limit is arbitrary, it establishes a time beyond which it is reasonable to undertake to determine whether a person who has become a persistent carrier harbors virulent bacilli or only harbors avirulent ones, in order that needless isolation and restriction may be avoided" (43). Brooks and Godfrey (44) state that "approximately 95 per cent. of cultures from recovered cases and contact carriers, tested for virulence up to three months from date of onset, have shown virulence." They see no indication for virulence tests in this group before the above period has elapsed. On the other hand, they advocate the immediate testing of morphologically positive cultures from non-contact carriers, whether these carriers are discovered in groups where diphtheria has been prevalent, or not. The policy outlined by Brooks and Godfrey is followed by the New York State Department of Health, whose regulations further provide that restrictions may be removed from a carrier of organisms not virulent for guinea pigs. Hartley and Martin (45), in a study of 457 convalescents, have shown that the rate of disappearance of *B. diphtheriae* from the throat can be expressed mathematically, but that this rate varies with the number of negative cultures required to determine freedom from diphtheria bacilli. In their work three successive negative cultures at weekly intervals were required. Approximately five per cent. of those remaining each day after the fifth day became free from the bacilli in 24 hours. If only two negative cultures had been required the rate would have been 5.4 per cent., and if only one negative culture had been required it would have been 7.1 per cent. On the basis of a five per cent. daily reduction, it follows that approximately 50 per cent. of the convalescents would still be carriers at the end of two weeks, 25 per cent. at the end of four weeks, and 10 per cent. at the end of six weeks following recovery. Weaver (46) found 28.8 per cent. of 500 convalescents to be carriers three weeks after onset, and 16.8 per cent. after four weeks. At the end of seven weeks less than one per cent. were still positive. The group studied by Weaver was composed of children in a hospital where constant care was given to irritant conditions, while the group of Hartley and Martin was composed of adults in a military camp. Weaver attributes the higher rate of disappearance of organisms, in his group, to this difference. If we accept the conclusions of Brooks and Godfrey, there is very little to be gained by virulence tests of convalescents until the carrier state has existed for three months. However, the rate of disappearance of diphtheria bacilli, as shown by Hartley and Martin, is so rapid that laboratories could well afford to make tests on the 25 per cent. of convalescent carriers persisting beyond the fourth week following recovery. Even if the virulence rate should be high in this group, the individual patient would be satisfied that every effort was being made to release him at the earliest possible moment.

In the autumn term of 1920 five cases of diphtheria appeared in the Berkeley High School, and cultures were taken from 1,441 persons representing the entire school population. In this typical non-contact group 223 carriers were found. Cultures from a few of these carriers were obtained by us from the Berkeley Health Department and tested for virulence with uniformly negative results. These 223 carriers were excluded from school and readmitted on the basis of freedom from diphtheria bacilli in two successive negative cultures. It will be seen from the curve (Fig. 1), representing the percentage distribution of these carriers, by days, from diagnosis to release, that 37.1 per cent. were still carriers at the end of two weeks, 10.8 per cent. at the end of three weeks and 4.2 per cent. at the end of four weeks. This indicates a more rapid rate of disappearance of bacilli from non-contact carriers than the one determined by Hartley and Martin for convalescents. According to the administrative procedure of New York State virulence tests would have been made on this entire group of 223 carriers representing 15.5 per cent. of the school population. This is, of course, the most rational procedure, for there is no legal excuse for the exclusion of the carrier of avirulent diphtheria bacilli from school for even a day. However, the rate of disappearance was so rapid that, had virulence tests been made, the amount of work thrown on the laboratory would have been materially lessened, if the tests had been postponed for two weeks from the date of diagnosis.

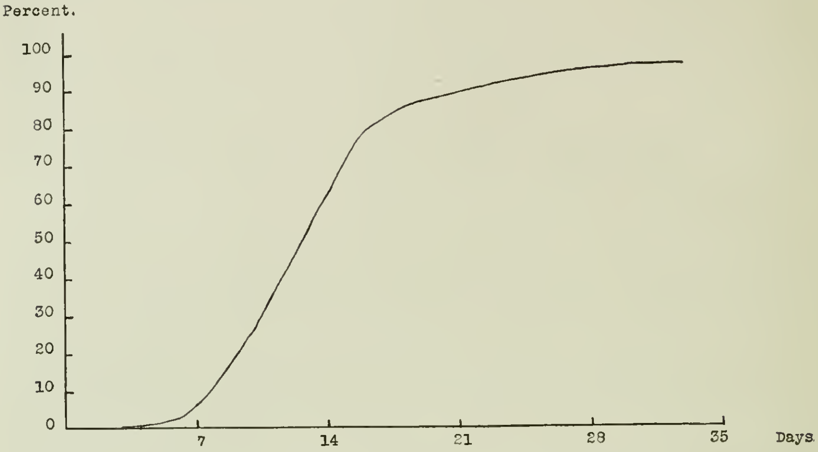

Fig. 1. Cumulative percentage distribution of 223 diphtheria carriers by days from diagnosis to release on two negative cultures.

It is believed that the amount of time consumed in routine examination of cultures would be decreased and more time found for virulence tests, if field cultures were taken more advisedly. We have a tremendous amount of evidence that virulent organisms are relatively uncommon in non-contact or indefinite contact groups. The taking of routine cultures from such groups should be considered a last resort, to be employed only when diphtheria persists in the group in spite of careful culturing of known contacts. In other words, the relation of virulence to the carrier state appears to be as follows:

1. Convalescent carriers usually harbor virulent organisms.
2. Contact carriers may or may not harbor virulent organisms.
3. Non-contact carriers usually harbor avirulent organisms.

A more extensive administrative use of the virulence test would undoubtedly hasten the return of many carriers now unjustly excluded from school, under the rule demanding two successive cultures, showing no organisms morphologically indistinguishable from diphtheria bacilli.

VIII. Summary.

1. The literature on methods of performing virulence tests of *B. diphtheriae* and on the results of such tests is reviewed.

2. A method for testing the virulence of *B. diphtheriae* by means of the intracutaneous inoculation of guinea pigs with field cultures is described.

3. Virulence tests, made by this method on cultures of *B. diphtheriae* from various sources, are compared with tests of the same cultures made by the subcutaneous method, described by Wayson.

4. It is demonstrated that the intracutaneous virulence test with field cultures is less often influenced by contaminating organisms than the subcutaneous test with field cultures.

5. It is further shown that the occasional difficulties of interpretation of the intracutaneous test, due to contaminating organisms, are negligible, when compared with the saving in time and animals made possible by the intracutaneous inoculation of field instead of pure cultures.

6. The results of virulence tests made with field cultures from various sources are compared with the results of virulence tests with pure cultures from similar sources.

7. The more extensive use of the virulence test in the administrative control of diphtheria is discussed, especially with reference to the prevention of loss of school time by children who may be carriers of avirulent diphtheria bacilli.

The authors wish to express appreciation of the assistance rendered them in this investigation by Miss Eschscholtzia Lichthardt and Miss Dorothy Beck. The virulence tests of the 15 pure cultures were made by Miss Adriana Jongeneel and Miss Jean Johnston who also isolated these cultures from the corresponding field cultures.

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EXPLANATION OF PLATES.

Plate XVII.

Intracutaneous virulence test. Photographed 48 hours after inoculation with positive field cultures 5 and 6 from subjects with clinical diphtheria. The normal animal (*N*) shows necrosis at both inoculation sites. The animal protected by antitoxin (*A*) shows slight traumatic effects of the needle puncture.

Plate XVIII.

Intracutaneous virulence test. Photographed 72 hours after inoculation with positive field cultures 12 (left) and 11 (right) from subjects with clinical diphtheria and simple tonsillitis respectively. The normal animal (*N*) shows a necrotic area, which is beginning to be replaced by a crust, at the site of inoculation of culture 12 and shows slight trauma at the site of culture 11. The animal protected by antitoxin (*A*) shows slight redness and induration at the site of culture 12 and slight trauma at the site of culture 11.

Plate XIX.

Intracutaneous virulence test. Characteristic "target" appearance of lesion 48 hours after inoculation with a positive field culture from a subject with clinical diphtheria.

Plate XX.

Intracutaneous virulence test. Lesions produced in a normal and in an antitoxin-protected animal by inoculation with a field culture of moderate virulence. 1, 2, 3: First, second and third day appearance in normal animal. 1′, 2′, 3′: Appearance on corresponding days in protected animal.

Plate XXI.

Intracutaneous virulence test. Lesions produced in a normal and in an antitoxin-protected animal by inoculation with a field culture of high virulence and with one containing avirulent organisms. The lesion produced by the virulent culture in the normal animal progressed until the death of the animal. The lesion produced by the avirulent culture retrogressed after the second day corresponding to the lesions produced by the same cultures in the protected animal. 1, 2, 3: Second, third and fourth day appearance in normal animal. 1′, 2′, 3′: Appearance on corresponding days in protected animal.

PLATE XVII.

PLATE XVIII.

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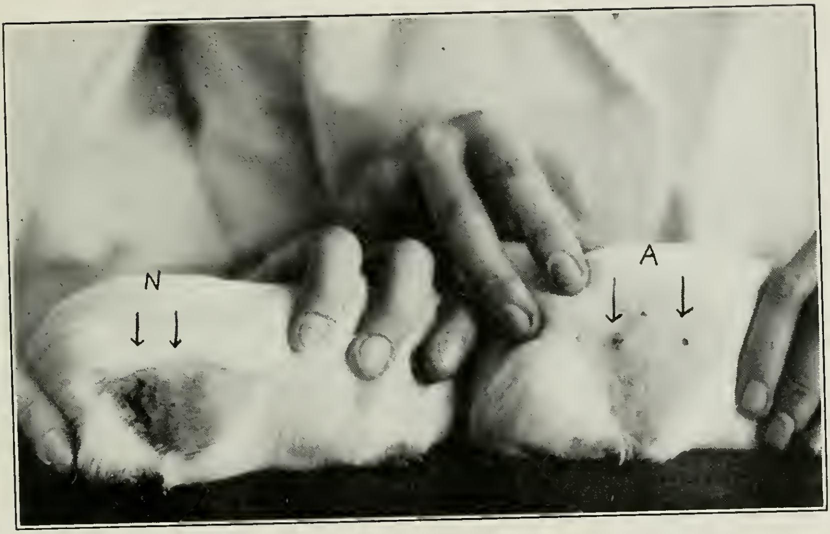

PLATE XIX.

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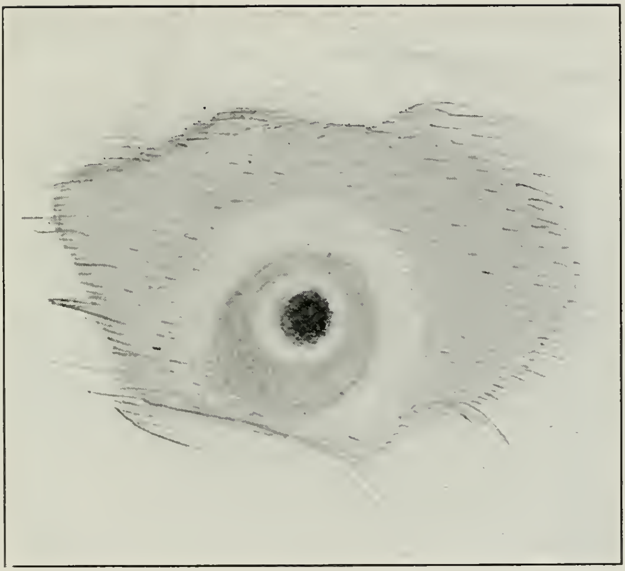

PLATE XX.

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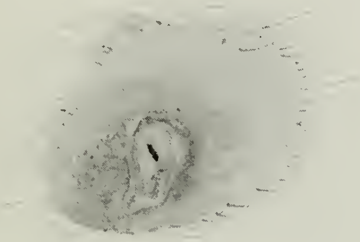

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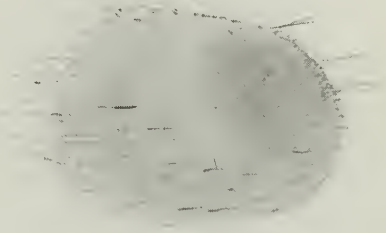

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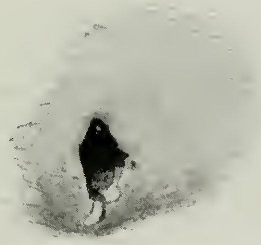

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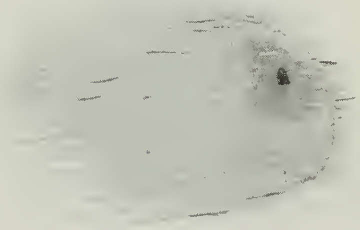

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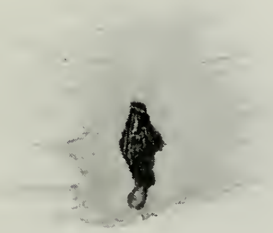

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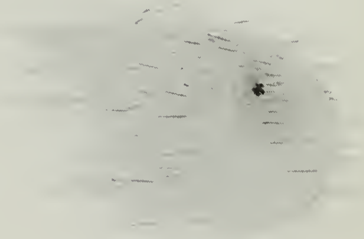

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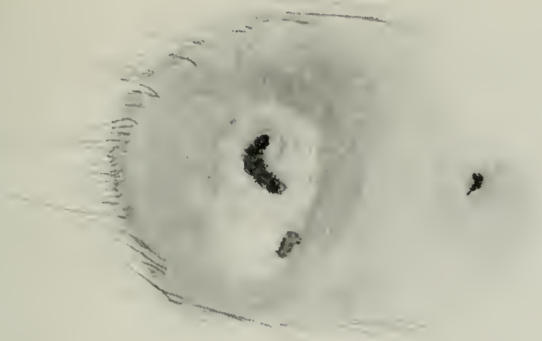
PLATE XXI.



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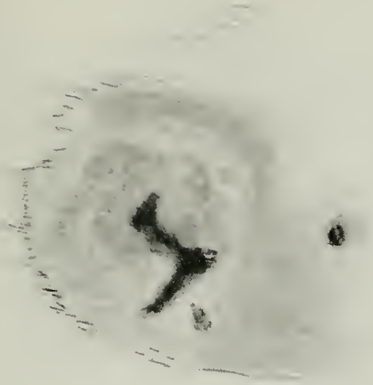

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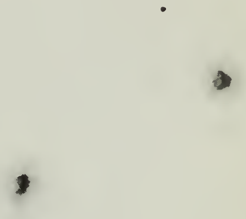

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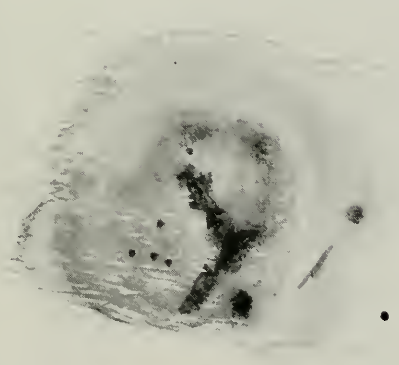

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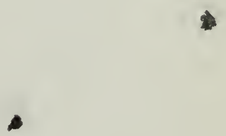

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THE RELATION BETWEEN FATIGUE AND THE SUSCEPTIBILITY OF GUINEA PIGS TO INFECTIONS OF TYPE I PNEUMOCOCCUS.*

By EDITH E. NICHOLLS and REYNOLD A. SPAETH.

(Received for publication, April 28, 1922.)

PRELIMINARY.

Fatigue is quite generally believed to increase the susceptibility of individuals to disease. In a recent paper Cowles ('18) has reported that at the Groton School "in several instances boys had been discharged from the infirmary after respiratory infections and returned soon after with pneumonia" (p. 556). In a single case: "A boy who had been in the infirmary with fever, coryza and a cough went out convalescent, took a long cross-country run, came in tired and quickly developed pneumonia." A study of the infirmary records showed twenty cases of pneumonia between 1906 and 1909 "confined to boys given to athletic tasks and sports." Cowles states specifically that "the kind of fatigue, observed as a cause of pneumonia at Groton, is an acute fatigue, due to violent exercise like rowing or running, competitive sports, and not mere weariness from everyday activities."

H. M. Vernon ('21) has advanced statistical evidence showing fatigue arising directly from industrial work to be "one of the most important contributory causes of sickness" (p. 143). Men who worked excessively long hours whether at heavy or light labor showed a higher percentage of sub-normal health than their fellow workers who kept shorter hours.

After reviewing the evidence Vernon admits that but little of it indicates the influence of fatigue on sickness. Such a relation is certainly suggested but far from proven. The interpretation of industrial statistics is notoriously difficult. Sickness is frequently given as a facile excuse for lost time. When working hours are reduced "sickness" drops off also, but this fact scarcely warrants the conclusion that the physical fatigue of the long hours is a cause of sickness. In this connection Vernon states (l. c., p. 7) "the men at a government ordnance factory lost 7.0 per cent. of their time from sickness, when they were working 63 and a half hours per week, but only 4.0 per cent., when the hours were reduced to 54 a week. The women at the same factory lost 4.3 per cent. of their time from sickness when they were on a 54-hour week, but only 2.8 per cent. of it, when on a 44 and a quarter hour week. Hence there appeared to be a direct relationship between the amount of sickness and the duration of working hours."

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* From the Physiological Laboratory, School of Hygiene and Public Health, Johns Hopkins University.

Health of munition workers in relation to weekly hours of work.
(Vernon, l. c., p. 169.)

	Weekly hours of work.	Very heavy or heavy labor.		Medium or light labor.	
		Number of workers in group.	Percentage with subnormal health.	Number of workers in group.	Percentage with subnormal health.
Men aged 41 or more	Under 70	190	21	203	23
Ditto	Over 70	147	31	82	33
Boys aged 18 or less	Under 60	165	5	162	8
Ditto	Over 60	62	11	1,010	10

The experimental evidence of the relation between acute fatigue (physical exhaustion) and susceptibility to infections has been surprisingly meager. In a recent paper Miss Oppenheimer and one of us (R. A. S., '22) have reviewed this experimental evidence, and have furthermore shown that fatigue, artificially induced in white and hooded rats by forcing them to run in motor-driven drums, definitely increases the resistance of the rats to intraperitoneal injections of lethal doses of Type I pneumococcus. These results are so at variance with the generally accepted view that it seemed possible, either (1) that they might be peculiar to rats, or (2) that they might involve some obscure technical error. We have therefore carried out a similar series of experiments with guinea pigs. Our results have corroborated the previous findings in rats. Guinea pigs may be definitely protected against lethal infections of Type I pneumococcus by forcing them to run in motor-driven drums. Physical exhaustion therefore decreases susceptibility to Type I pneumococcus, both in white and hooded rats and in guinea pigs.

EXPERIMENTAL MATERIAL.

Our guinea pigs were obtained from dealers. Previous to experimentation they were kept under observation for ten days or two weeks, or until they showed by their weight curves a satisfactory adaptation to their new environment. They were kept in cages of the U. S. Hygienic Laboratory type, and were fed a mixed diet of hay, oats, fresh greens, milk and water. We used pigs weighing from 150 to 300 grams. These weights correspond approximately to an age of one to two months on Minot's ('91) growth curve.

APPARATUS.

We used the apparatus of Oppenheimer and Spaeth (l. c.). In order to prevent the pigs' catching their long hind toes in the wire mesh, the drums were partly wound with tire tape. As in the case of rats, some pigs learned to run very quickly, while others floundered and slid about in a bewildered and helpless fashion. We had little difficulty, however, in exhausting the pigs, even though they failed to solve the problem of running. As in the rat experiments, the animals were considered exhausted, when they lay down in the drums and failed to respond to ordinary exciting stimuli, such as pounding on the table, tapping on the drums and gentle handling. Exhaustion is not a function of the number of drum revolutions. It varies with individual pigs and with the extent of their mastery of the running problem.

The pigs were always weighed immediately before and after having been run. They always lost weight, but unlike the rats they did not regain this weight-loss on the following day. Each animal was subjected to three running periods, and the injections of pneumococci were given either before or after running.

VARIATIONS IN THE SUSCEPTIBILITY OF PIGMENTED AND ALBINO GUINEA PIGS TO TYPE I PNEUMOCOCCUS.

At the outset of our experiments we attempted to determine a minimal lethal dose for our culture of pneumococci. We tried to establish a dosage, independent of the weights of the animals. This procedure, which had been successful with rats, proved quite hopeless in the case of guinea pigs. At least two variables complicated the results. After a long series of preliminary experiments, in which we were working in the dark, we found that constant results could be obtained by giving a definite dose per gram weight. At once it became evident that albino guinea pigs were more susceptible to our culture than were the pigmented pigs. This difference in susceptibility was of the order of 300 per cent. It is clearly shown in Fig. 1 where all the unfatigued control pigs that were used in our subsequent experiments have been charted. A dose of 0.0005 c.c. of culture, per gram weight, was lethal for albino pigs, while 0.0016 c.c. was necessary to kill pigmented pigs.

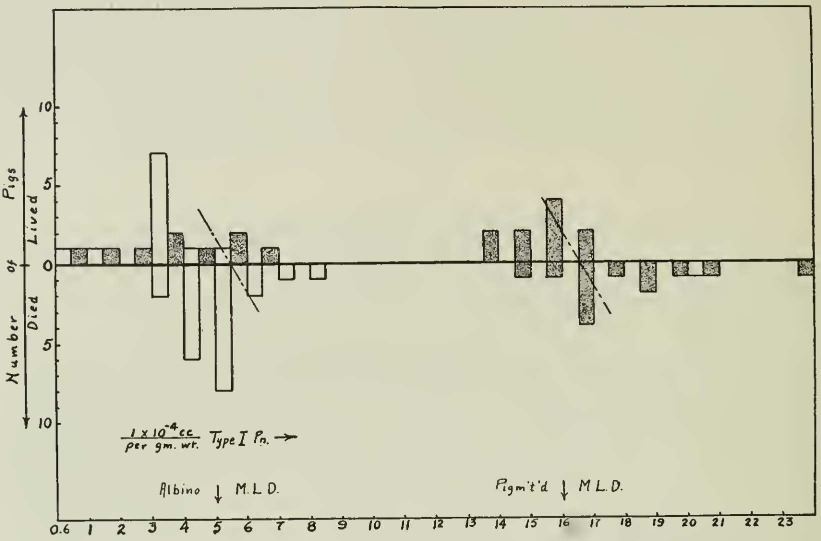


Fig. 1. The difference of susceptibility to Type I pneumococcus in albino and pigmented guinea pigs is shown in this chart. The heavy abscissa represents dosage increase from left to right. Ordinates above 0 are pigs that lived; below 0 are pigs that died. Albinos are shown as white rectangles; pigmented pigs as stippled rectangles. A dosage of 0.0005 c.c. per gm. wt. is fatal to albinos, but in this region all pigmented pigs live. Not until the dosage becomes 0.0016 c.c. per gm. wt. do all pigmented animals die. Albino guinea pigs are therefore about three times as susceptible to Type I pneumococcus as are pigmented pigs. Sixty-three animals are represented in the chart. The virulence of the pneumococcus culture was constant throughout the experiments.

The maintenance of a constant virulence in Type I pneumococcus is not a simple matter. From our previous experience with this organism we concluded that for our experiments a culture of minimal virulence was preferable. We therefore selected a culture (2) that had never, to our knowledge, been through a guinea pig. The virulence of this culture was apparently at base-level. We had no difficulty in maintaining it constant by transplating to fresh blood-broth medium according to the scheme of Table I.

TABLE I.

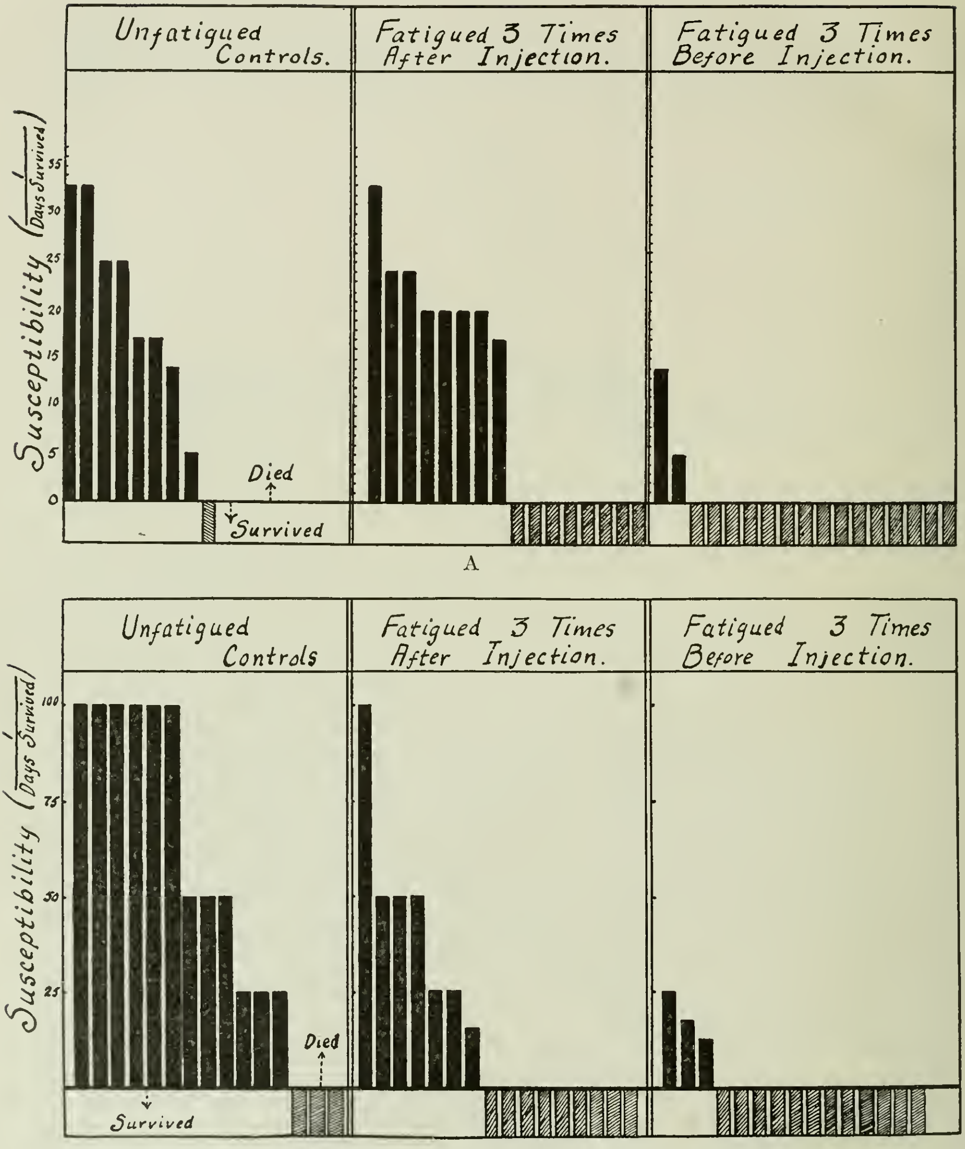
No. of Transplants	*Original Culture*		
	↓		
1)	24 hours incub.		
	↓		
	3–4 days on ice.		
	↓		
2)	—24 hours incub.		
	↓		
3)	18 hours incub.	———→	to pigs
	→3–4 days on ice.		
	↓		
3a)	—24 hours culture		
	↓		
4)	18 hours culture	———→	to pigs
etc.	→3–4 days on ice.		

METHODS OF CONTROL.

In the experiments of Oppenheimer and Spaeth (l. c.) on rats, none of the experimental animal stock died of extraneous infections. Nevertheless autopsies were not systematically made, nor was the pneumococcus organism recovered in the dead animals. In our experiments with guinea pigs we therefore made systematic autopsies and smears of the heart's blood, pleural and peritoneal exudates. In all doubtful cases, or where secondary infections were indicated, the heart's blood was grown on blood agar. Only deaths that were unequivocally attributable to pneumococcal infections are included in our tabulated results.

RELATION BETWEEN FATIGUE AND PNEUMOCOCCAL INFECTIONS IN GUINEA PIGS.

Our experimental results are shown in Fig. 2, *B*. In the unfatigued control group 80 per cent. of the animals died, while in the case of animals fatigued three times before injection, 80 per cent. lived. As in the case of the rat experiments of Oppenheimer and Spaeth (l. c.) fatigue after injection protected the guinea pigs less



A

B

FIG. 2. The relation between fatigue and susceptibility to Type I pneumococcus is shown in these charts for rats (*A* from Oppenheimer and Spaeth (l. c.)) and guinea pigs (*B*). The similarity in the two charts is unmistakable. Fatigue before injection (three periods of physical exhaustion) definitely increases the resistance of both rats and guinea pigs to lethal doses of Type I pneumococcus. This decrease of susceptibility is indicated by the diminution of the black areas from left to right in both charts. See text for details.

TABLE II.

Date.	Color.		Weight of pig in grams.	Dose per gm. wt.	Dose in c.c.	Fatigued.		Un-fatigued controls.	Death.
	Albino.	Pig-m't'd.				Before.	After.		
Dec. 21, '21........	*	–	257	0.0005 c.c.	0.13	*	–	–	–
	*	–	241	"	0.12	–	*	–	–
	*	–	163	"	0.08	–	*	–	4th day
	*	–	301	"	0.15	–	–	*	1st "
	*	–	191	"	0.09	–	–	*	1st "
Jan. 18, '22........	–	*	239	0.0016 c.c.	0.38	*	–	–	–
	–	*	185	"	0.30	*	–	–	6th day
	–	*	202	"	0.32	–	*	–	2d "
	–	*	230	"	0.37	–	–	*	1st "
	–	*	192	"	0.30	–	–	*	2d "
Jan. 25, '22........	*	–	158	0.0005 c.c.	0.08	*	–	–	–
	*	–	193	"	0.09	*	–	–	–
	*	–	158	"	0.08	–	*	–	4th day
	*	–	221	"	0.11	–	*	-	–
	*	–	154	"	0.07	–	–	*	–
	*	–	173	"	0.08	–	–	*	4th day
Feb. 1, '22.........	–	*	228	0.0016 c.c.	0.36	*	–	–	4th day
	–	*	216	"	0.34	*	–	–	–
	–	*	173	"	0.28	–	*	–	1st day
	–	*	231	"	0.31	–	*	–	6th "
	–	*	207	"	0.33	–	*	–	–
	–	*	170	"	0.27	–	–	*	–
	–	*	216	"	0.34	–	–	*	1st day
Feb. 22, '22........	*	–	224	0.0005 c.c.	0.11	*	–	–	–
	*	–	209	"	0.10	*	–	–	–
	*	–	231	"	0.11	*	–	–	–
	*	–	206	"	0.10	–	*	–	2d day
	*	–	195	"	0.09	–	*	–	–
	*	–	192	"	0.09	–	*	–	–
	*	–	166	"	0.08	–	–	*	4th day
	*	–	203	"	0.10	–	–	*	1st "
Mar. 3, '22........	–	*	241	0.0016 c.c.	0.38	*	–	–	–
	–	*	225	"	0.36	*	–	–	–
	–	*	272	"	0.43	–	*	–	–
	–	*	288	"	0.46	–	*	–	–
	–	*	220	"	0.35	–	–	*	–
	–	*	199	"	0.32	–	–	*	4th day

Experimental data of animals plotted in Fig. 2, B.

well than fatigue before injection In this group nine of the sixteen pigs (56 per cent.) lived while seven (44 per cent.) died.

The essential corroboration of our results of the findings on rats by Oppenheimer and Spaeth (l. c.) is shown by comparing *A* and *B* in Fig. 2. The relations between pneumococcus infection and fatigue are given both for rats and guinea pigs. The most striking difference between the response of guinea pigs to pneumococci and that of rats is the much shorter incubation period in the pigs. Sixty per cent. of the control pigs died within two days after the injection, whereas, with a minimal lethal dose, no control rats died before the third day. Forty-six pigs are included in our chart. Of this number 28 were albinos and 18 were pigmented. The albinos and pigmented pigs received quite different doses of pneumococci (vide infra), but each group was independently controlled for its particular minimal lethal dose (see Table II).

General conclusions.

1. There is in guinea pigs a definite correlation between pigmentation and susceptibility to Type I pneumococcus. White-coated, pink-eyed pigs, which are probably genetically pure albinos (3), were found to be approximately 300 per cent. more susceptible to a given culture of Type I pneumococcus than were pigmented pigs.

2. Fatigue, artificially induced in white or pigmented guinea pigs by forcing them to run in motor-driven drums, definitely increases the resistance of these pigs to lethal injections of Type I pneumococcus. This occurs whether fatigue precedes or follows the pneumococcal injections, but is distinctly more effective in the case of fatigue before injection.

3. The experimental results of our study corroborate the work of Oppenheimer and Spaeth (l. c.) on the relation between fatigue and susceptibility to Type I pneumococcus in white and hooded rats.

Notes.

1. From the Physiological Laboratory of the School of Hygiene and Public Health, Johns Hopkins University.

2. We are indebted to Dr. C. G. Bull and to Miss Clara McKee of the Department of Immunology for our original pneumococcus culture, for the standardized media used throughout the experiments and for much invaluable advice.

3. It is necessary to make this reservation, since Sewall Wright ('16) has found a white-coated, pink-eyed guinea pig that does not breed true. This type of "somatic albino" may be produced by combining two other factors with dilute sepia. Whether susceptibility to pneumococcus infection is correlated with the genetic or the somatic white pig, or with both, can easily be shown experimentally. Oppenheimer and Spaeth (l. c.) found no difference in susceptibility to pneumococcus between white (albino) and hooded rats. Lewis and Wright ('21) likewise reported no difference between the susceptibility of white and pigmented guinea pigs to tuberculosis. Guinea pigs, therefore, offer a unique opportunity to test the relation between natural resistance and an inherited character-albinism. With Dr. Wright's collaboration the problem is under investigation.

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PATHOLOGICAL EFFECTS OF DIPHTHERIA TOXIN IN THE GUINEA PIG, WITH SPECIAL REFERENCE TO A LESION OF BOWMAN'S CAPSULE.*

By IDA W. PRITCHETT, Sc.D.

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Plates XXII to XXVII.

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The study of the pathology of experimental diphtheria dates well back into the nineteenth century, having developed in close association with the study of the pathology of the disease in human beings. The earliest attempts to cause the disease in animals were, naturally enough, directed toward the production of pseudo-membranes in the throat. Bretonneau (6), who gave the malady its present name, attempted to produce false membranes in dogs with irritant chemical substances, but recognized that the membranes so formed, and the clinical conditions invoked, were not identical with those seen in human cases of diphtheria. He had previously failed to reproduce the disease in animals by the inoculation of material obtained from patients, but in spite of these early failures the belief gradually grew up that the disease was of a specifically infectious nature. In 1883 Klebs (20) demonstrated in the typical pseudo-membrane a characteristic bacillus which he was unable to isolate in pure culture. This, however, was accomplished in 1884 by Loeffler (23), who was able to produce typical lesions in guinea pigs and rabbits, rats and mice proving to be refractory. Loeffler believed that a toxic substance secreted by the bacilli was the true cause of the lesions and of death, and in 1887 and 1888 demonstrated such a toxic substance in glycerine extracts of meat in which the bacillus had been grown. It remained, however, for Roux and Yersin (38), in 1888, to obtain a cell-free toxic filtrate from broth cultures of the bacilli which, though very weak as judged by present day standards, was capable of producing, on inoculation into susceptible animals in doses of thirty-five c.c., the pathological effects characteristic of this toxin.

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* From the Department of Immunology of the School of Hygiene and Public Health of the Johns Hopkins University.

The lesions described in human and animal intoxications have been many and varied. In 1887 Oertel (30) published his monograph, including many plates showing lesions observed in human cases, with special reference to the false membrane in the throat and to the necrotic foci, with pyknosis and karyorrhexis of the cell nuclei, encountered in the lymphoid tissue. Lesions following the injection into animals of live cultures or of cell-free toxin have been described as occurring in almost every tissue of the body, notably at the site of inoculation in the subcutaneous tissue, in the adrenals, liver, heart, lungs, kidneys, spleen and other lymphoid structures, mucous and serous surfaces and nervous tissue. Since it is of interest to know how the more important lesions are related to each other in regard to the time required for their development, it seems worth while briefly to summarize the chief lesions hitherto described and to note, as far as possible, their order of development as observed in the experimental animals employed in the present study.

Experimental lesions due to diphtheria toxin.

It has been long since recognized that the lesions caused by the diphtheria bacillus are identical with those produced by the cell-free toxin, with the exception of the pseudo-membrane. Dogs, cats, rabbits, guinea pigs, birds, rats, mice, sheep, cattle and horses have all been used for the study of the action of this toxin. Of these, rats and mice have been found to be relatively refractory, the other animals showing varying degrees of susceptibility.

The chief lesions described may be briefly summarized as follows:

1. *Skin and underlying muscle, following subcutaneous inoculation.*—Pseudo-membranes are not formed under the skin after the

injection of cell-free toxin, but instead there is massive gelatinous oedema of the subcutaneous and intermuscular connective tissue, later becoming hemorrhagic and leading eventually to necrosis and sloughing. Alopecia is frequently described, Cobbett (7) noting it even in rats, which were refractory to any but very massive doses of toxin, and showed no local necrosis from subcutaneous injections.

2. *Adrenals.*—Great congestion and hemorrhage, in severe cases extremely destructive. Occasionally, however, this lesion is slight even in fatal intoxications. Roux and Yersin (39) note that "almost constantly" the adrenals are red in guinea pigs dying of the infection.

3. *Liver.*—Extensive fatty degeneration has been cited by Welch and Flexner (43, 44), Flexner (14) and others. These authors also note focal necroses, surrounded by areas of hemorrhage, varying in size from a few necrotic cells to foci easily seen with the naked eye. Flexner (14), working with guinea pigs, rabbits and kittens, observed that such areas were largest and most frequent in the guinea pig, and occurred only in animals dead of infections, or of bacterial products or similar substances, being almost unknown in normal guinea pigs. Congestion of the liver is also noted.

4. *Heart.*—Fatty infiltration, fragmented nuclei and degeneration of the muscle fibers have all been described. Flexner (14) observed that the fatty infiltration and nuclear changes were most marked in rapidly fatal cases. Park (32), in describing human autopsy findings, mentions degeneration of the muscle fibers, swelling of the vascular endothelium and hemorrhages as being characteristic of late cases, the rapidly fatal ones showing only slight parenchymatous changes. Jaffé (17), working with diphtheria infection in guinea pigs, noted that parenchymatous changes, consisting of a granular opacity or albuminous degeneration, appeared early and were observed in almost all cases, fatty degeneration in about 2/3 of all cases. Homogeneity and myolysis were seldom noted, true waxy degeneration never. Interstitial changes were also observed.

5. *Lymph nodes and lymphoid tissue in general, including the spleen.*—The fragmentation of nuclei in the thymus, the germinal centers of lymph nodes and the Malpighian corpuscles of the spleen, described by Oertel (30) in human cases, has also been observed in experimental intoxications. Flexner (14) notes that there is more or less relation between the degree of concentration of the toxin and the extent of these lesions. Phagocytosis of the nuclear fragments by

large mononuclear cells has been observed. Hemorrhages into the lymphoid tissue may occur, and phagocytosis of the red cells is seen.

6. *Kidneys.*—Changes in these organs, causing albuminuria and nephritis, were noted in dogs by Roux and Yersin in 1889. Microscopically there have since been described hemorrhages; degeneration of all types of epithelium, with hyalinization of, and droplet formation in the cells of the convoluted tubules; cloudy swelling of the tubular epithelium; desquamation of the epithelial cells; thrombosis and rupture of the glomerular capillaries; swelling, desquamation and proliferation of the cells of Bowman's capsule; fatty degeneration of the tubules; albuminous material in the tubules and the subcapsular space; round-cell infiltration of the interstitial tissue, etc.

7. *Lungs.*—Oedema and scattered hemorrhages into the alveoli are sometimes described. Hydrothorax has been frequently observed postmortem.

8. *Mucous and serous surfaces.*—Hemorrhages are often noted in the mucous membrane of the stomach and intestines, as well as in the peritoneum and pleura.

9. *Brain and spinal cord.*—The lesions here are described by Flexner (14) and by Berkley (3) as consisting of small thickenings on the dendrites of the cortical and hippocampal cells. Changes in the Nissl substance of the cells of the anterior horn of the cord have been noted by Rainy (37).

EXPERIMENTAL.

Lesions observed in the tissues of "normal" guinea pigs, taken from stock.

It has been necessary to examine, as controls, tissues from a number of normal guinea pigs, and in the course of this work it has been found that many supposedly normal animals have, among other things, kidneys that are far from normal. Before proceeding further, it seems worth while brefly to mention these and other abnormalities encountered in the tissues of guinea pigs taken from stock.

1. *Pathological kidneys in "normal" guinea pigs.*—The most striking condition, and one frequently met with, is a chronic interstitial nephritis, often very extensive. It varies from small areas of round cell infiltration to large patches, sometimes visible to the naked eye in the stained section, in which often the tubules have become shrunken and no longer take the acid stain, showing here and

there in their contricted lumens groups of necrotic cells. Such a "normal" kidney is shown in Plate XXII, Figs. 1 and 2. Since this condition is so frequently encountered in the kidneys of "normal" guinea pigs, its occurrence in those that have received diphtheria toxin has been considered of little importance in the study of the effect of this toxin upon guinea pig kidneys. Mallory (25) notes that the interstitial nephritis observed frequently in human cases of diphtheria, at autopsy, seems to be independent of the epithelial degeneration, while the degeneration found in intense foci of infiltration appears to be due to malnutrition produced by the blocking of the blood vessels.

Apparently many so-called normal guinea pigs are subject also to a tubular nephritis, if one may judge from the marked proliferative process sometimes observed in these animals. Three sections of the "normal" kidney shown in Plate XXII exhibited the following marked proliferation (Table I).

TABLE I.

"Normal" guinea pig 5.	Mitoses seen in three sections of one kidney of a "normal" guinea pig.					
	Collecting tubules.	Convoluted tubules.	Bowman's capsule.	Glomerular tufts.	Connective tissue.	Total.
Section 1...	13	4	0	0	2	19
" 2...	6	4	0	0	0	10
" 3...	3	7	0	0	2	12
Total......	22	15	0	0	4	41

This is a fairly marked case, but others equally marked have been observed. The fact that such extensive regeneration of tubular epithelium may be encountered in supposedly normal guinea pigs throws doubt upon the same process in guinea pigs that have been injected with diphtheria toxin. Reference to Tables V, VI, VII and X will show that in these guinea pigs also proliferation of tubular epithelium was sometimes fairly pronounced, and irregularly so, long before other and more definite indications of toxic action could be detected. While it is probably true that in guinea pigs with marked local toxin lesions some of the proliferation of the cells of the tubules observed in the kidneys is in response to the specific toxic injury, it seems hardly allowable, in view of these findings in "normal" guinea pigs, to attribute it to the same cause in animals that have received mildly toxic or neutral doses. In such cases one can not escape the conclusion that the proliferation of tubular epithelium may be due to a pre-existing toxic agent of unknown nature, and one is therefore not justified in attributing it to the effects of the injected material.

2. Another curious condition sometimes encountered in "normal" guinea pigs is the heavy infiltration of the connective tissue with eosinophilic cells. These may be found in great numbers in the subcutaneous tissue and intermuscular connective tissue of the abdomen, the loose areolar tissue about the bronchi in the lung, the intestinal submucosa, the kidney pelvis, the auricles and root of the aorta and elsewhere. They occur in small numbers in the lymph nodes, general connective tissue and vessels of all guinea pigs, notably about the bronchi and the kidney pelvis, but are unusual in such masses as are occasionally encountered in supposedly normal animals. Plate XXIII, Fig. 1 shows a section through the skin and abdominal wall of a "normal" guinea pig taken from stock. A large clump of eosinophilic cells is shown in the loose connective tissue around a blood vessel. The whole section is filled with masses of these cells, many of them much larger than the one shown. It is interesting to note that the same guinea pig had a fairly marked degree of proliferation in the cells of the kidney tubules (13 tubular mitoses in the left kidney and 14 in the right, two sections of each kidney being examined), and that both kidneys showed a fair amount of round cell infiltration, while eosinophilic cells occurred in large numbers in the connective tissue everywhere. Similar cells are seen in great numbers in areas of old organized pneumonia in guinea pigs.

The etiologic agent in the interstitial nephritis of supposedly normal guinea pigs is, of course, obscure. The patches of round cell infiltration may represent old areas of healed pyelonephritis, due possibly to the small, Gram-negative bacillus of the hemorrhagic septicemia group which so often attacks guinea pigs and rabbits. It is by no means certain that the accumulation of eosinophilic cells in the areolar tissue, and the interstitial nephritis and proliferation of tubular epithelium observed are parts of the same morbid process, but their simultaneous occurrence in cases such as this suggests that they may all owe their presence to some relatively mild chronic intoxication.

3. *Congestion of the viscera.*—While this can, perhaps, not be called a distinctly pathological condition, it is observed so commonly

in normal guinea pigs that its occurrence in those that have received diphtheria toxin must necessarily be regarded with a certain amount of distrust. The livers of guinea pigs frequently show very marked congestion, as do the kidneys, in which the glomerular capillaries and the large vessels are sometimes engorged. For this reason congestion of the viscera, especially of the liver and kidney, has been more or less disregarded in the present study, except when it was extreme, or occurred in organs not usually congested, or was accompanied by hemorrhage or frankly retrograde changes.

The order of development of the lesions due to diphtheria toxin as observed in the tissues of the guinea pig.

The tissues of the guinea pigs employed in this study have all been taken as soon as possible after death and fixed in Zenker's fluid. After being embedded in paraffin, they were cut at 5–7 microns and stained with Unna's alkaline methylene blue and eosin, then mounted in balsam for examination.

The development of the various lesions has been studied (1) in guinea pigs, each injected subcutaneously with one m.l.d. of toxin and etherized at intervals up to the fourth day; (2) in those dying acutely (24–48 hours) after subcutaneous injection of overwhelming doses of toxin; (3) in those dying acutely (under 24 hours) after massive intravenous doses of toxin; (4) in those receiving doses of toxin and antitoxin lying between the L-o and the L-+ mixtures. An effort has been made to correlate these various findings and to determine, from a study of both the chronic and acute intoxications, the order of development of the chief lesions in the various internal organs. The nervous system has not been included in this study.

The following description of the lesions encountered in the organs of the guinea pig after administration of diphtheria toxin is based chiefly on a study of such organs taken from guinea pigs each injected subcutaneously with 1 m.l.d. of toxin, the animals being killed at intervals up to four days. Sections were made from the local lesion in the skin and abdominal wall, the adrenals, liver, heart, lungs, kidneys, spleen and mesenteric and other lymph nodes. A brief description of the microscopic pathology of such sections is here given.

1. *Abdominal wall with its overlying skin, the site of inoculation.*—Oedema is massive even at 24 hours, polymorphonuclear infiltra-

tion is fairly heavy and there may be some slight necrosis of the skin. At 36–48 hours hemorrhage becomes apparent, leukocytic infiltration is heavier and there is increasing necrosis. At about 48–72 hours the oedema reaches its height and begins to recede. The rest of the story is simply one of deepening necrosis and leukocytic infiltration of the whole abdominal wall. If death does not occur too early (7–10 days with doses of toxin below the m.l.d.), there may be dry necrosis and sloughing.

2. *Adrenals.*—Roughly speaking, the hemorrhage seems to begin at about 36 hours and to increase up to 48–72 hours, receding thereafter. It is usually most marked in guinea pigs that have received large doses of toxin, when it may be so extreme as to disorganize the tissue and nearly to rupture the capsule, but when death is very rapid (18–24 hours), or delayed up to or beyond 4 days, it may be slight or absent, having failed to occur at all in the first case and having, in all probability, cleared up in the second. Regeneration in the cells of the adrenal, following massive hemorrhage, may be very marked in 48 hours, the zona fasciculata and zona glomerulosa showing, in the order named, the greatest tendency to proliferation, the zona reticularis and the medulla practically none. Enlargement of the adrenal is usually noted, and may be seen even when there is little or no gross hemorrhage or congestion, due perhaps to an accumulation of fluid.

3. *Liver.*—Congestion commonly occurs and may even go on to hemorrhage. A very few isolated necrotic cells may be seen even at 24 hours, their number increasing thereafter. Flexner (14) observed that these necrotic areas seemed to occur less frequently on the periphery of the lobule than toward its center. In the present study they have been seen inconstantly in the gross material, but were sometimes as large as 1–2 mm. in diameter, and one case was encountered in which almost half the liver tissue appeared to be coagulated. Marked regeneration may occur in the liver cells, the proliferative process beginning at some time between 48 and 72 hours and declining after 72 hours. During the same period some of the liver cells take on a vacuolated appearance, due to the deposition of fat droplets. These vacuolated cells increase in number until they appear to form a network over the section, and at 96 hours or later they can be seen to lie chiefly in the center of the lobule, sparing the portal area. The same vacuolated appearance has been met with in the livers of guinea pigs dying acutely (48 hours), and was very

marked in some paralytic animals killed four weeks after injection. A brief resumé of the liver findings is given in Table II. The figures are taken from one experiment, but are considered typical of all. One section of each liver was examined.

TABLE II.

Hours lived.	Foci of necrosis.	Congestion.	Fatty infiltration.	Mitoses observed.	
				Liver cells.	Kupfer cells.
24, killed....	Very few cells	Marked	None seen	0	0
36, "	A few cells	"	" "	0	1
48, "	Small foci	Very marked	Slight?	0	1
72, "	" "	Slight	Some	41	1
96, " ...	Few, 1 large	Marked	Much, central	10	0
103, died.....	Several, small	Marked, also hemorrhage	" "	8	0

4. *Heart.*—Nothing but slight capillary congestion was noted up to 72 hours, when occasional hyaline-looking muscle fibers began to be encountered. Fat stains were not done, but at 96 hours and after there was some vacuolation of the muscle fibers suggestive of fatty infiltration. Flexner (14) notes that fatty metamorphosis of the heart is rarely absent, and is most marked in rapidly fatal cases.

5. *Lungs.*—As a rule, nothing of interest was encountered. When hydrothorax was present, congestion and oedema of the lungs were seen. When it was absent, as often happened, there was nothing particularly characteristic observed in the lung tissue.

6. *Spleen.*—Congestion was slight at 24 hours, but by 48–72 hours sometimes assumed the proportions of an acute splenic tumor, receding somewhat thereafter. Phagocytosis of red cells in the pulp was slight up to 48 hours, but occurred in the rest of the series. The typical pyknosis and karyorrhexis of cells in the lymphoid nodules, described by Oertel (30), was never very marked in a series such as this, being most pronounced in rapidly fatal cases (15–48 hours), especially if the toxin had been given intravenously, when fragmentation of nuclei was observed throughout the organ. In the present series the lymphoid lesion was encountered to some degree at 24

hours and reached its height at 48, decreasing at 72 hours and being almost completely absent at 96. The nuclear fragments were taken up by large pale cells, probably endothelial leukocytes, which also take up the red blood cells in the pulp. At 72 hours some large cells suggestive of megakaryocytes were seen in the pulp, but no nucleated red cells were observed.

A brief summary of the findings in the spleen is given in Table III. One section from each guinea pig was examined.

TABLE III.

Hours lived.	Congestion.	Lymphoid lesion.	Phagocytosis of red cells.	Remarks.
24	Slight	Slight	None seen	Nuclear debris intracellular
36	Increased	Increased	" "	" " "
48	Acute splenic tumor	Greatest	Beginning	Debris intra- and extra-cellular
72	" " "	Decreased	Continued	Megakaryocytes? No nucleated reds
96	Receding?	Going	"	
103	Still fairly marked	Gone	"	

7. *Lymph nodes.*—The mesenteric nodes showed some central congestion more or less throughout the series. Phagocytosis of red cells began to be noticeable at 48–72 hours, and was thereafter more prominent than in the spleen. Pyknosis and karyorrhexis of cell nuclei in the germinal centers extended over a rather wider zone than in the spleen, being sometimes fairly marked at 36 hours and lasting through 72 hours, with the high point again at 48 hours. As in the spleen, this lesion was found to be most pronounced in rapidly fatal intoxications (15–48 hours), especially if the toxin had been given intravenously, when fragmentation of nuclei occurred throughout the node.

The clearing up of the lymphoid lesion described above is not in agreement with the observations of Welch and Flexner (44), who record the autopsy findings in a guinea pig dying three weeks after a second subcutaneous dose of diphtheria toxin. In this guinea pig the changes were most intense in the axillary and inguinal nodes, and consisted of fragmentation of the nuclei and phagocytosis of the debris by large pale cells. The axillary and inguinal nodes were not examined regularly in the present experiments, nor was the effect of repeated injections tried. Flexner (14), using subcutaneous injections, observed that the nodes most closely related to the seat of inoculation showed the greatest changes. In his experiments the time elapsing between the inoculation and death varied from 18 hours to 2 weeks, and he states that the pathological lesions, except as regards intensity, were not influenced by the strength of the toxin or the period of incubation when the latter was within this limit of time. It is certain that different lymph nodes and lymphoid structures from the same animal show, in many cases, very different degrees of pyknosis and karyorrhexis, and it may be that the axillary and inguinal nodes would have shown these changes persisting at a time when they were disappearing in the mesenteric nodes and the spleen. Possibly, however, an examination of the mesenteric lymph nodes, and other lymphoid tissues even farther removed from the seat of inoculation, gives a better index of the general effect of the toxin upon this type of tissue than can be gained from an examination of the axillary and inguinal nodes, so close to the point of injection on the abdomen and exposed perhaps more than the other lymphoid tissues to the full force of the toxin. Such examinations, made in the present series of experiments, indicate that the lymphoid lesion in guinea pigs, at least in its marked and decisive form, is characteristic of rapidly fatal intoxications rather than of more chronic ones, and that the debris is very quickly cleared away by large phagocytic cells.

8. *Kidneys.*—Definite pathological changes did not become apparent until 48 hours, when they consisted of small hyaline droplets in the epithelium of the convoluted tubules, chiefly in that situated just under the fibrous capsule of the kidney; some solidly staining hyaline convoluted tubules; a few small casts, either hyaline or composed of desquamated epithelial cells; occasional bits of hyaline fibrin in the glomerular capillaries; some albuminous material in the tubules and the subcapsular space (also observed earlier, and sometimes in supposedly normal guinea pigs); occasional slight thickening of Bowman's capsule, which, however, was not very definite before 72 hours, and did not become marked until 96 hours, at which time proliferation was observed in the thickened cells. The retrograde changes were progressive, becoming extremely pronounced at 96 hours and after. Hypertrophy of some of the uninjured glomeruli was observed to replace those thrombosed, ruptured or otherwise incapacitated. Toward the end of the series congestion became pronounced to the point of engorgement and hemorrhage. A fuller

TABLE IV.

Development of lesions in the organs of guinea pigs injected subcutaneously with 1 m.l.d. of diphtheria toxin and etherized at intervals up to four days.

Hours lived.	Abdominal wall and overlying skin.	Adrenals.	Liver.	Heart.	Kidneys.	Spleen.	Lymph nodes.
24.....	Massive oedema. Marked leukocytic infiltration.	No hemorrhage. Little or no proliferation.	Almost no necrosis. No proliferation or fat.	Slight capillary congestion.	No retrograde changes or proliferation. Bowman's capsule normal.	Congestion and lymphoid lesion slight.	Slight central congestion and lymphoid lesion.
36.....	Same, increased and showing hemorrhage and necrosis.	Slight hemorrhage. Little proliferation.	A few necrotic cells. No proliferation or fat.	Same.	A few casts? No retrograde changes. Little proliferation. Capsule normal.	Congestion and lymphoid lesion increased.	Slight central congestion. Lymphoid lesion increased.
48.....	Same. Necrosis increasing.	Hemorrhage most marked? Little proliferation.	Small foci of necrosis. Some fat deposit. No mitoses.	Same.	A few casts. Hyaline degeneration begun. Little proliferation. Capsule normal.	Acute splenic tumor. Greatest lymphoid lesion.	Same. Greatest lymphoid lesion. Phagocytosis of red cells.
72.....	Same. Oedema at its most massive point.	Hemorrhage receding?	Small foci of necrosis. Some fat deposit. Proliferation most marked.	Hyaline muscle fibers occasionally seen.	Many casts. Fibrin in glomerular loops. Marked degeneration. Capsule slightly thickened.	Same. Lymphoid lesion decreasing. Red cells phagocyted.	Lymphoid lesion decreasing. Red cells phagocyted.
96.....	Oedema receding slightly. Necrosis increasing.	Hemorrhage patchy. Slight proliferation.	Small foci of necrosis. Much fat becoming central. Less proliferation.	Same. Vacuolated fibers suggest fat.	Same. Capsule thickened and proliferating.	Congestion and lymphoid lesion less. Red cells phagocyted.	Lymphoid lesion almost gone. Red cells phagocyted.
103 (Died)	Same. Skin growing hard.	Same.	Same. Also hemorrhages.	Same.	Same.	Still congested. Lymphoid lesion gone.	Lymphoid lesion gone. Red cells phagocyted.

description of the lesion encountered in Bowman's capsule is given below.

The pathological changes observed in the tissues of 250 gram guinea pigs injected subcutaneously with 1 m.l.d. of diphtheria toxin and killed at intervals up to 4 days are briefly condensed in Table IV.

Lesions seen in Bowman's capsule following the injection of (a) toxin and (b) toxin-antitoxin mixtures.

The kidney lesions have proved to be the most interesting part of the present experiments, especially the lesion observed in Bowman's capsule. This consists, as already briefly stated, of a swelling and proliferation of the capsular cells, giving them a marked prominence in kidneys where the condition is well advanced. In extreme cases the cells assume almost the appearance of cuboidal epithelium, the nuclei becoming round instead of flat, while in properly stained sections the whole cell is distinctly basophilic. This very striking effect is usually not obtained until comparatively late. It is seldom seen until the third day after injection, and then only to a moderate degree, there being some swelling but no proliferation. By the fourth day, however, it is usually well marked, both in fatal cases (i.e., those receiving the m.l.d. of toxin or the L+ dose of toxin-antitoxin), and in non-fatal cases with distinct local toxin lesions killed at that time (Plate XXIV, Fig. 2). By 96 hours proliferation of the capsular cells may be considerable. On the other hand, in some cases the dose has been too overwhelmingly toxic, and the cells may be greatly swollen, but show little or no proliferation. In such cases the injury has been too great to allow of immediate regeneration, though it is possible that if death does not intervene too rapidly regeneration may follow later. Marked swelling and proliferation of the capsules has been observed at least four weeks after injection of severely toxic doses of toxin-antitoxin, and in the same kidneys were seen many capsules with fairly normal, flat nuclei packed together end to end as closely as they would go. No tendency to fibrosis of the capsule or obliteration of the capsular space has been noted.

It is conceivable that a quantity of toxin sufficient to cause a marked lesion at the point of inoculation should also be sufficiently toxic to cause lesions in the internal organs. Indeed this is probably true of every case of human diphtheria that goes on to recovery, and although the lesions may not persist as scars within the organs, they could, in all probability, be demonstrated during the acute stage of the disease and perhaps for some time after. But it is somewhat surprising to find that doses of toxin and antitoxin so balanced that they just fail to cause a local lesion (i.e., the L-o dose), or those causing only a very slight and fleeting lesion, are still capable, in many instances. of producing in guinea pigs, although to a less marked degree, the same proliferation and swelling of Bowman's capsule that is seen with sublethal or minimal lethal doses of toxin alone. In such kidneys the retrograde changes observed in severe intoxications are wanting. The tubular epithelium is not necrotic and hyaline, casts are seldom or never seen, the kidney is apparently normal except for thickening of some of the capsules of Bowman and occasionally some proliferation of their cells. An instance is shown in Plate XXV, Fig. 2. Here may be seen a glomerular tuft and its capsule. The cells of the capsule are distinctly swollen and prominent, and at one point (*A*) is a large, clear cell in mitosis. This guinea pig had received a mixture of toxin and antitoxin lying just about at the L-o dose. It had no gross lesion at any time, and when killed on the fourth day after inoculation showed, in sections taken through the inoculated area on the abdominal wall, nothing abnormal (Plate XXV, Fig. 1). The guinea pig next above this in the series showed a very slight and fleeting local lesion at the point of inoculation, with little manifestation of injury visible in sections taken through the skin and abdominal wall. Plate XXVI, Fig. 2 shows a group of glomeruli from the kidney of this guinea pig (146, Table V), almost every one of which has a decidedly thickened and prominent capsule. It is surprising to see such marked and widespread capsular thickening resulting from so mildly toxic a dose. Plate XXVI, Fig. 1 shows a section taken through the skin and abdominal wall of this guinea pig. The only thing which may perhaps be considered indicative of toxic action is the slight, patchy extravasation of blood just under the epidermis, and the congestion of the small subcutaneous blood vessels, neither of them shown very well in the picture. This section may be compared with that shown in Plate XXIV, Fig. 1, taken from a guinea pig which had received a large dose of pure toxin and was killed on the fourth day (kidney from this guinea pig shown in Plate XXIV, Fig. 2). Here there is a very massive oedema of the subcutaneous tissue, sparsely infiltrated with polymorphonuclear leukocytes. The skin is necrotic and thickened, the underlying muscle oedematous, somewhat disorganized and infiltrated with leukocytes.

A protocol showing the development of capsular lesions in the kid-

TABLE V.

Lesions and proliferation observed in the kidneys of guinea pigs receiving subcutaneous injections of toxin-antitoxin run far below the L-o dose, the mixtures incubated for 30 minutes at room temperature before being injected.

The doses of toxin varied from .1 c.c. to .31 c.c., falling at intervals of .03 c.c. The L-o dose lay at .19 c.c., the L-+ at .33 c.c. All guinea pigs were killed on the fourth day after injection. Two sections of each kidney examined.

Guinea pig.	Weight in grams.	Gross skin lesion.	Casts.	Collecting tubules.	Convoluted tubules.	Bowman's capsule.	Glomerular tufts.	Remarks.
142......	245	None.	Few, pyramid of left.	Right, 0 Left, 1	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	No necrosis. Bowman's capsule normal.
143......	245	None.	None seen.	Right, 0 Left, 3	Right, 1 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	No necrosis. Bowman's capsule normal.
144......	250	None.	None seen.	Right, 3 Left, 3	Right, 3 Left, 4	Right, 0 Left, 0	Right, 0 Left, 0	No necrosis. Bowman's capsule normal.
145......	250	None.	One in the left.	Right, 1 Left, 2	Right, 1 Left, 2	Right, 2 Left, 2	Right, 0 Left, 0	No necrosis. Some capsules thickened.
146......	250	Slight.	None seen.	Right, 3 Left, 5	Right, 2 Left, 2	Right, 0 Left, 7	Right, 0 Left, 0	Fairly widespread thickening of capsules.
147......	250	Slight oedema.	A few, small.	Right, 1 Left, 1	Right, 1 Left, 2	Right, 5 Left, 5	Right, 0 Left, 0	Same. Capsules are increasingly thick.
148......	260	Oedema.	Some, small.	Right, 4 Left, 4	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Same. Capsules markedly thickened.
149......	265	Oedema.	A good many.	Right, 7 Left, 11	Right, 0 Left, 2	Right, 1 Left, 0	Right, 0 Left, 1	Same. Marked retrograde changes.

neys of guinea pigs following the subcutaneous injection of exactly or nearly neutral doses of toxin-antitoxin is given in Table V.

A comparison of Plate XXV, Fig. 2 and Plate XXIV, Fig. 2 will show that in spite of the very great difference in the toxicity of the two doses injected into these guinea pigs, as evidenced by their effect on the tissues at the site of inoculation, the capsular lesion in their kidneys differs only in extent. In both, Bowman's capsule is thickened and proliferated, more intensely and far more universally so in that of the guinea pig receiving the frankly toxic dose. Another instance of the effect of diphtheria toxin on Bowman's capsule is shown in Plate XXIII, Fig. 2, taken from the kidney of a guinea pig which had received a subcutaneous dose of toxin insufficient to kill on the fourth day, but lethal on the sixth. This shows a markedly thickened capsule in which there is a large mitosis (*A*). Indications of more prolonged and intense toxic action are present here in the hyaline casts visible at (*B*) and (*C*). The kidney is markedly necrotic throughout.

It would probably be an exaggeration to say that this swelling and proliferation of Bowman's capsule is seen in every guinea pig of 250 grams which receives, subcutaneously, the L-o mixture of toxin and antitoxin and is killed on the fourth day. In this, as in all other reactions of the animal organism, there is individual variation. It is well known that guinea pigs of the same weight, if given the same sublethal dose of toxin, will not develop equally marked local lesions, nor will they succumb in the same length of time to its chronic effects, a few animals even surviving doses fatal for others of equal weight. If, however, the right balance is struck between the susceptibility of the guinea pig's tissues, local and general, and the degree of neutralization of the toxin-antitoxin mixture injected, the effect described above can be obtained. No comparable swelling and no proliferation of the capsules has ever been seen in a normal guinea pig, nor in one which had received subcutaneously a toxin-antitoxin mixture lying below the L-o dose, or in one which had received subcutaneously a quantity of toxin too small to cause a slight local lesion. Occasionally in normal guinea pigs, especially those with a marked chronic interstitial nephritis, there may be seen here and there a somewhat swollen capsule, especially on the boundary between cortex and medulla, where they sometimes appear to be surrounded by fibrous tissue. But, as already stated, proliferation has never been seen in these capsules, nor are they ever numerous.

Duration of the lesion in Bowman's capsule.

A single experiment was run, including eight guinea pigs, to study the duration of the capsular lesion in these animals. The injections in this series were given subcutaneously, and consisted of 1 c.c. of a toxin-antitoxin immunizing mixture, obtained from the New York City Board of Health. These mixtures, being slightly toxic, sometimes produce in guinea pigs, even in 1 c.c. quantities, a very slight local thickening which has practically disappeared by the fourth or seventh day. The first guinea pig was killed four days after injection, the second at seven days and the others at weekly intervals, the whole experiment lasting over a period of fifty days. Since these mixtures are used to immunize children, it seemed of interest to investigate their action, although in such relatively enormous quantities, on the kidneys of the highly susceptible guinea pig. It was found that throughout the series Bowman's capsule showed a very slight thickening, never very pronounced or even very definite, certainly not comparable in severity to that previously recorded. The lesion apparently tended to disappear without scarring, no fibrosis of the capsule being observed. A protocol of the kidney findings is given in Table VI. Guinea pigs injected with 2–3 c.c. of this immunizing mixture developed a slight subcutaneous oedema at the point of inoculation and showed, when killed on the fourth day, distinct thickening of some of the capsules of Bowman and occasionally slight proliferation of their cells.

It is difficult to keep alive over a long period a series of guinea pigs that have received a severely toxic dose of filtrate. They gradually waste away and die of late paralysis or of some intercurrent infection. For this reason it is almost impossible to follow the fate of the capsular lesion in severe intoxications over a longer period than one or two weeks. This might perhaps be done if the severely toxic doses were later supplemented by doses of antitoxin. In two unusually resistant guinea pigs, injected with toxin-antitoxin mixtures closely approaching the L-+ dose, and killed on the 27th day when completely paralyzed and struggling for breath, the capsules of Bowman were still markedly and extensively thickened, and showed occasional proliferation. The exact fate of the capsular lesion, in its most pronounced form, remains to be determined.

It may be of interest to compare the tubular and capsular proliferation noted in the foregoing protocols in guinea pigs receiving

TABLE VI.

Duration of the lesion in Bowman's capsule, following the subcutaneous injection of 1 c.c. of a toxin-antitoxin immunizing mixture.

Guinea pig.	Weight in grams.		Days since injection.	Mitotic figures observed.				Remarks.
	Original.	Final.		Collecting tubules.	Convoluted tubules.	Bowman's capsule.	Glomerular tufts.	
150......	250	230	4	Right, 0	Right, 0	Right, 0	Right, 0	An occasional slightly thick capsule.
				Left, 0	Left, 1	Left, 0	Left, 0	
151......	255	260	7	Right, 0	Right, 0	Right, 2	Right, 0	Same. A few small casts.
				Left, 2	Left, 1	Left, 0	Left, 0	
152......	255	315	14	Right, 1	Right, 2	Right, 1	Right, 0	An occasional slightly thick capsule.
				Left, 2	Left, 3	Left, 1	Left, 0	
153......	260	370	21	Right, 0	Right, 0	Right, 0	Right, 0	Same.
				Left, 1	Left, 4	Left, 0	Left, 0	
154......	260	330	28	Right, 1	Right, 0	Right, 0	Right, 0	Same.
				Left, 2	Left, 4	Left, 0	Left, 0	
155......	265	425	36	Right, 0	Right, 5	Right, 0	Right, 0	Same, several casts.
				Left, 0	Left, 3	Left, 0	Left, 0	
156......	265	480	43	Right, 0	Right, 2	Right, 0	Right, 0	Slight residual thickening of capsule. Some casts.
				Left, 0	Left, 0	Left, 0	Left, 0	
157......	265	400	50	Right, 1	Right, 10	Right, 0	Right, 0	Very slight residual thickening of capsule.
				Left, 0	Left, 5	Left, 0	Left, 0	

graded doses of toxin and antitoxin with that observed in a series of guinea pigs injected subcutaneously with graded doses of toxin alone. In such a series the retrograde changes are usually very intense. One such series is recorded in Table VII, in which the doses of toxin ranged from 1/25000 c.c. to 1/200 c.c. The development of the skin lesion and of the capsular lesion may here be followed. It may be noted that the injury and proliferation observed at the end of this series is more extensive than that recorded in the preceding tables where toxin-antitoxin mixtures were used, but that the difference is only one of degree. The inconstant occurrence of chronic interstitial nephritis in guinea pigs is well demonstrated. It may also be noted that while proliferation of the tubular epithelium in the last three guinea pigs is so marked and progressive as to be, in all probability, due to the action of the toxin, fairly marked proliferation is present even in those with the smallest doses of toxin, and occurs inconstantly thereafter until doses of marked toxicity are reached. On the other hand, evidences of injury to Bowman's capsule do not appear until after a slight local lesion is produced, and proliferation in its cells, once established, is more or less progressive and at the end very marked. Only in guinea pig 137 was there extreme necrosis of all the kidney elements. As in the preceding series, proliferation in the glomerular tufts was not observed. This is usually not encountered until the fifth or sixth day after the injection of severely toxic doses.

That the lesions of Bowman's capsule described above are due to the whole toxin or some fraction of it, and not either to some nonspecific, thermostable constituent of the broth or to some injurious substance in the antitoxic globulin solution, may be shown by heating the toxin to 75° C. for five minutes before mixing it with the antitoxin. In parallel series of guinea pigs injected with heated and unheated toxin, mixed with antitoxin and incubated thirty minutes at room temperature (the mixture made up in each case to a 2 c.c. volume with salt solution), the characteristic capsular lesion was obtained in the series receiving the unheated toxin, but not in the other. The conditions of the two experiments were identical except for the heating of the toxin. The protocol is given in Table VIII.

Some observations on two dissimilar toxins.

The experiment recorded in Table VII was repeated with a different toxin. The toxin used in Table VII, and in all the other experi-

TABLE VII.

Series of guinea pigs injected subcutaneously with graduated doses of diphtheria toxin approaching the m.l.d.

All the animals were killed on the fourth day after injection. Two sections of each kidney examined, except in the case of guinea pig 132, in which only one section of each was seen.

Guinea pig.	Weight in grams.	Toxin dose.	Gross skin lesion.	Kidneys: Lesions observed.			Kidneys: Mitoses observed.			
				Round cell infiltration.	Casts.	Bowman's capsule.	Collecting tubules.	Convoluted tubules.	Bowman's capsule.	Glomerular tufts.
129...	225	$^1/_{25000}$ c.c.	None.	Some.	A few.	Normal.	Right, 5 Left, 4	Right, 4 Left, 2	Right, 0 Left, 0	Right, 0 Left, 0
130...	235	$^1/_{20000}$ c.c.	None.	Some.	A few.	Normal.	Right, 0 Left, 1	Right, 3 Left, 2	Right, 0 Left, 0	Right, 0 Left, 0
131...	235	$^1/_{15000}$ c.c.	Slight and fleeting.	Some.	None seen.	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
132...	245	$^1/_{10000}$ c.c.	Slight.	Some.	None seen.	Normal.	Right, 0 Left, 0	Right, 1 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
133...	245	$^1/_{7000}$ c.c.	Slight oedema.	None seen.	None seen.	Normal.	Right, 0 Left, 0	Right, 1 Left, 1	Right, 0 Left, 0	Right, 0 Left, 0
134...	245	$^1/_{5000}$ c.c.	Slight.	Fairly marked.	None seen.	Slightly thickened.	Right, 0 Left, 1	Right, 0 Left, 1	Right, 0 Left, 0	Right, 0 Left, 0
135...	245	$^1/_{1000}$ c.c.	Marked.	Fairly marked.	A few.	Moderately thickened.	Right, 8 Left, 14	Right, 4 Left, 4	Right, 1 Left, 3	Right, 0 Left, 0
136...	250	$^1/_{500}$ c.c.	Very marked.	None seen.	A good many.	Markedly thickened.	Right, 13 Left, 13	Right, 1 Left, 3	Right, 2 Left, 1	Right, 0 Left, 0
137...	260	$^1/_{200}$ c.c.	Very marked.	Little if any.	Very many.	Markedly thickened.	Right, 17 Left, 30	Right, 7 Left, 8	Right, 6 Left, 14	Right, 0 Left, 0

TABLE VIII.

Guinea pigs injected with toxin-antitoxin in parallel series, in which toxin heated to 75° C. for five minutes is compared with unheated toxin for its action on the kidneys.

Toxin mixed with one unit of antitoxin and incubated for thirty minutes at room temperature before injection. All guinea pigs killed four days after injection. Two sections of each kidney examined.

Guinea pig.	Weight in grams.	Toxin content of mixture.		Gross skin lesion.	Kidneys: Lesions observed.			Kidneys: Mitoses observed.		
		Heated.	Unheated.		Round cell infiltration.	Casts.	Bowman's capsule.	Collecting tubules.	Convoluted tubules.	Bowman's capsule.
168...	245	.15 c.c.		None.	Little or none.	None seen.	Normal.	Right, 0 Left, 0	Right, 1 Left, 0	Right, 0 Left, 0
169...	255	.2 c.c.		None.	Little or none.	None seen.	Normal.	Right, 1 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
170...	260	.25 c.c.		None.	Little or none.	A few, small.	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
171...	265	.3 c.c.		None.	Little or none.	A few, small.	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
172...	240		.15 c.c.	None.	Fairly marked.	A few, small.	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
173...	245		.2 c.c.	None.	Fairly marked.	None seen.	Slightly thick.	Right, 0 Left, 1	Right, 0 Left, 0	Right, 0 Left, 0
174...	255		.25 c.c.	Slight.	Slight.	None seen.	Moderately thick.	Right, 0 Left, 1	Right, 2 Left, 1	Right, 0 Left, 1
175...	270		.3 c.c.	None.	Fairly marked.	A few, small.	Markedly thick.	Right, 0 Left, 5	Right, 5 Left, 6	Right, 1 Left, 0

ments, unless otherwise stated, was prepared on October 5, 1920, with a transplant of *B. diphtheriae* Park 8, obtained at that time from the New York City Board of Health. I shall designate this as Toxin 1. The second toxin was prepared February 8, 1922 with a second transplant of *B. diphtheriae* Park 8, obtained later from the same source, and this I shall designate as Toxin 2. During the first six months of its use, covering the period in which most of the recorded experiments were performed, Toxin 1 showed the following measurements:

M.L.D.	.004 c.c.
L-o dose	.19 c.c.
L-+ dose	.33 c.c.
L-+ minus L-o	35 m.l.d.

Toxin 2, during the first two months of its use, showed the following measurements:

M.L.D.	.0035 c.c.
L-o dose	.11 c.c.
L-+ dose	.15 c.c.
L-+ minus L-o	about 11 m.l.d.

These are approximate measurements, since it was sometimes necessary, in order not to use quite so many guinea pigs, to adopt intervals of .02 or .03 c.c. in determining the L-o and L-+ doses, instead of the usual interval of .01 c.c. Nevertheless, such error as exists is not large, and since the conditions of the two sets of experiments were the same, may be disregarded. The important point is that the measurements clearly indicate that the two toxins differed materially not only in potency, but also, and to a more marked degree, in their toxon fraction, Toxin 1 showing a difference of 35 m.l.d between the L-o and L-+ doses, Toxin 2 only about 11 m.l.d. This difference is of interest in view of the results obtained when graded doses of Toxin 2, ranging from 1/30000 c.c. to 1/300 c.c., were injected subcutaneously into guinea pigs. If the results recorded in Table VII, where Toxin 1 was used, are compared with those in Table IX, where Toxin 2 was used, it will be seen that Toxin 2, though somewhat more potent, was incapable of causing either a local lesion in the skin or any swelling and proliferation in Bowman's capsule in quantities as small as 1/1000 c.c., whereas Toxin 1 produced a very marked skin lesion at 1/1000 c.c. and a slight skin lesion even at 1/10000 or 1/15000 c.c., while at 1/5000 c.c. a slight thickening of Bowman's capsule could be detected. It is unfortunate that a dilution of 1/3000 c.c. was not run with Toxin 1, as it would probably have shown a marked lesion of the capsule. However, it appears from the figures presented that Toxin 1, though somewhat less potent than Toxin 2, had, at a comparable age, almost 25 times its power of causing a detectable lesion when injected under the skin of a medium-sized guinea pig, and between 6 and 8 times its power of causing a lesion in Bowman's capsule when so injected. As noted also in connection with the experiment recorded in Table X, where a new and hitherto untried antitoxin was used, the tendency of Toxin 2 to produce comparatively little local reaction on subcutaneous injection into guinea pigs was a disturbing factor in the exact determination of its L-o dose.

Some intracutaneous tests were run in guinea pigs with both toxins, to see how their relative toxicity compared by this method. When these experiments were performed, Toxin 1 was about 18 months old, Toxin 2 about 2 months old. The m.l.d. for Toxin 1 at this time lay in the neighborhood of .0055 c.c., while that of Toxin 2 was, as already noted, .0035 c.c. Nevertheless, intracutaneous tests of the two toxins, done in the usual manner with 1/500 of the dilution which was to be tested (a .2 c.c. volume being injected in each case to allow for slight and unavoidable loss during the injection), indicated that Toxin 1 should have had an m.l.d. of about 1/300 c.c., while Toxin 2 should have killed, on subcutaneous injection, in a dilution of about 1/250. As has been stated, the two toxins actually showed quite the reverse of these findings, Toxin 1 failing to kill on the fourth day in a dilution of 1/300 even when it was new. These experiments bear out absolutely the findings recorded previously, in which it was found that the less potent toxin was in reality far more able to produce necrosis at the point of injection than was the more potent one.

These findings are, to say the least, paradoxical. It has been supposed that necrosis at the site of injection was caused by the specific toxin fraction of the filtrate, yet here we have a toxin slightly inferior in toxicity which is able to produce oedema and necrosis at the site of injection in far higher dilution than a more potent one. Furthermore, a distinct capsular lesion is caused in the kidneys of guinea pigs by the less powerful toxin in dilutions in which the stronger toxin is without effect. A large number of toxins must be studied in order to determine whether this property is a peculiarity of this special toxin, or may be encountered in other toxins which

TABLE IX.

Series of guinea pigs injected subcutaneously with graduated doses of diphtheria toxin approaching the m.l.d. All animals killed on the fourth day after injection; none died. One section of each tissue examined.

Guinea pig.	Weight in grams.	Toxin dose.	Gross skin lesion.	Kidneys: Lesions observed.			Kidneys: Mitoses observed.			
				Round cell infiltration.	Casts.	Bowman's capsule.	Collecting tubules.	Convoluted tubules.	Bowman's capsule.	Glomerular tufts.
389...	245	$^1/_{30000}$ c.c.	None.	None seen.	None seen.	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
390...	245	$^1/_{25000}$ c.c.	None.	A little.	None seen.	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
391...	245	$^1/_{23000}$ c.c.	None.	Little or none.	None seen.	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
392...	250	$^1/_{20000}$ c.c.	None.	Some.	None seen.	Normal.	Right, 1 Left, 2	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
393...	250	$^1/_{17000}$ c.c.	None.	Little or none.	None seen.	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
394...	270	$^1/_{15000}$ c.c.	None.	A little.	A few?	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
395...	275	$^1/_{10000}$ c.c.	None.	Some.	None seen.	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
396...	275	$^1/_{7000}$ c.c.	None.	Some.	None seen.	Normal.	Right, 1 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
397...	280	$^1/_{5000}$ c.c.	None.	Slight.	None seen.	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
398...	280	$^1/_{3000}$ c.c.	None.	Little if any.	None seen.	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
399...	280	$^1/_{1000}$ c.c.	None.	Very little.	A few?	Normal.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0
400...	280	$^1/_{600}$ c.c.	Marked oedema.	None seen.	A few.	Fairly thick.	Right, 0 Left, 1	Right, 0 Left, 1	Right, 1 Left, 0	Right, 0 Left, 0
401...	290	$^1/_{300}$ c.c.	Marked oedema.	None seen.	A good many.	Markedly thickened.	Right, 5 Left, 15	Right, 0 Left, 4	Right, 1 Left, 0	Right, 1 Left, 0

are notably deficient in the toxon fraction. The findings recorded above suggest that the capsular lesions encountered in the kidneys of guinea pigs after the injection of neutral, or nearly neutral, toxin-antitoxin mixtures may be caused by some fraction of the diphtheria filtrate other than the specifically toxic one, and that it is somehow bound up with the toxon content of the filtrate. Since it is the toxon that is supposed to cause the late paralyses so characteristic both of human and of animal diphtheria intoxications, it will be interesting to determine whether this toxon-deficient filtrate is able to cause late paralyses in guinea pigs, when injected in mixtures between the L-o and the L-+ doses, with the same readiness as toxins richer in toxon. Should it fail to do so, it would strongly indicate that the paralysis-producing substance and the substance causing capsular lesions are identical, or closely associated.

The results of the injection of Toxin 2 in dilutions of 1/30000 to 1/300 c.c. are given in Table IX.

Kidney lesions and fatal outcome in parallel series of guinea pigs injected (a) subcutaneously and (b) intravenously with toxin-antitoxin mixtures lying between the L-o and the L-+ doses.

This experiment was carried out with Toxin 2, already described in connection with the experiment recorded in Table IX. It was undertaken in an effort to determine whether mixtures of toxin and antitoxin lying between the L-o and L-+ doses, injected intravenously into guinea pigs, would cause a lesion in Bowman's capsule at a point of lower toxicity than would the same mixtures injected subcutaneously. It seemed probable that this would happen, since toxin-antitoxin mixtures which were easily withstood, when given under the skin, frequently proved fatal when given into the vein. This phenomenon has already been recorded by other workers, among them Morgenroth (29), who noted both in guinea pigs and rabbits that mixtures of diphtheria toxin and antitoxin, well borne when given subcutaneously, were fatal when given intravenously, observing that mixtures which, according to previous conception, contained only toxon, acted, on intravenous injection, as if they still contained free toxin.

As might have been expected, lesions of Bowman's capsule were encountered in the kidneys of guinea pigs that had received intravenously mixtures of toxin and antitoxin which failed to produce equally marked effects when given subcutaneously. Toxin 2 was somewhat difficult to compare with Toxin 1 for its ability to produce capsular lesions in neutral, or nearly neutral mixtures, since it caused so little oedema at the site of inoculation even in mixtures of toxin and antitoxin closely approaching the L-+ dose. It was even difficult to be sure exactly where the L-o dose lay, and it is possible that in the measurements of this toxin given above the L-o dose should be recorded as lying at about .12 c.c., thus reducing still further the toxon fraction. It seems certain, however, that marked capsular swelling, with proliferation, could be detected in the kidneys of guinea pigs injected intravenously with toxin-antitoxin mixtures which failed to produce an equally marked effect when given subcutaneously. The protocol of such an experiment is given in Table X. It will be seen from this table that slight capsular thickening, without proliferation, was produced in the kidneys of guinea pigs following their subcutaneous injection with toxin-antitoxin mixtures which failed to cause any lesion, gross or microscopic, at the point of inoculation, while the same mixtures, injected intravenously, caused marked capsular thickening with some proliferation. This would probably indicate either that a large part of the necessary toxic substance was absorbed by the subcutaneous tissue at the site of inoculation, or that it was unable to injure the capsule to the same extent when brought in contact with it little by little. It is unfortunate that heavier guinea pigs were not obtainable at this time, but it is doubtful if the use of heavier ones would have affected the results in any essential respect.

The harmful effects of diphtheria toxin upon Bowman's capsule have been noted by a number of writers, but usually rather briefly. Spronck (40) observed that in rabbits dying a few days after injection the epithelium of the capsule, as well as of the vascular loops, was changed and desquamating. Flexner (14) described contraction and necrosis of the nuclei of the capsule and the disintegration and desquamation of its cells. Babes (2) noted mitoses in the endothelium of the glomerular capillaries, in the capsular epithelium and in the cells of the labyrinth. Lyon (24) observed, in rapidly fatal cases in rabbits, that Bowman's capsule and its lining endothelium had not suffered much, the capsule showing swelling here and there, but the endothelium remaining intact and showing no proliferation or desquamation. In later cases also he noted that Bowman's capsule and its endothelium were not especially affected,

TABLE X.

Fatal outcome and lesions of Bowman's capsule occurring in parallel series of guinea pigs injected (a) subcutaneously and (b) intravenously with toxin-antitoxin mixtures lying between the L-o and the L-+ doses.

The antitoxin used in this experiment was not the same one previously used to standardize this toxin; the unit was simply accepted as that given on the container. One section of each kidney was examined. All non-fatal cases etherized at 4.5 days.

Guinea pig.	Weight in grams.	Toxin content of T-A mixture.		Gross skin lesion.	Kidneys: Mitoses observed.				Remarks.
		Subcutaneous.	Intravenous.		Collecting tubules.	Convoluted tubules.	Bowman's capsule.	Glomerular tufts.	
366...	220		.08 c.c.		Right, 14 Left, 4	Right, 0 Left, 2	Right, 0 Left, 0	Right, 0 Left, 0	Bowman's capsule normal. Little interstitial nephritis.
367...	225		.11 c.c.		Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Bowman's capsule normal. Little interstitial nephritis.
368...	225		.14 c.c.		Right, 0 Left, 0	Right, 1 Left, 1	Right, 0 Left, 4	Right, 0 Left, 0	Capsules markedly thickened. Little interstitial nephritis.
369...	225		.17 c.c.		....	....	....	....	Found dead, 65 hours. No sections made.
370...	245		.2 c.c.		....	....	....	....	Found dead, 42 hours. No sections made.
371...	210	.08 c.c.		None.	Right, 1 Left, 1	Right, 1 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Bowman's capsule normal. Fair interstitial nephritis.
372...	215	.11 c.c.		None.	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Right, 0 Left, 0	Bowman's capsule normal. No interstitial nephritis.
373...	215	.14 c.c.		None.	Right, 0 Left, 0	Right, 1 Left, 0	Right, 0 Left, 0	Right, 1 Left, 0	Slight capsular thickening and interstitial nephritis.
374...	220	.17 c.c.		Slight oedema.	Right, 7 Left, 46	Right, 1 Left, 5	Right, 6 Left, 5	Right, 0 Left, 1	Capsules markedly thickened. Little interstitial nephritis.
375...	220	.2 c.c.		Marked oedema.	....	....	....	....	Found dead, 87 hours. No sections made.

though the capsule might be swollen, homogeneous and occasionally fibrillated. Karsner and Denis (18) reported that, following repeated injections of small quantities of diphtheria toxin into rabbits, which were killed on the 31st day, the glomeruli showed well-marked swelling of the capsular epithelium, and in many cases slight proliferation of the cells. True epithelial crescents were not observed. Faber (12), using rabbits of 1,000–1,500 grams, injected intravenously, pointed out the small amount of damage to Bowman's capsule, the parietal layer being little altered. Later (13), working with rabbits injected first with small doses of diphtheria toxin and then with sublethal doses of *B. coli,* he noted that the parietal layer of Bowman's capsule usually remained intact, though it was occasionally slightly stratified. Oertel (30) and Park (32), in describing human autopsy findings, recorded swelling and proliferation of the capsular epithelium—precisely the condition observed in the guinea pig. On the whole, changes in Bowman's capsule seem not to have proved as interesting to investigators as those in the other elements of the kidney. The failure to observe capsular thickening in rapidly fatal cases can be readily understood on the basis of the findings in the present study. It may be that acute intoxications, rather than more chronic ones, have been most frequently dealt with in experimental work, so that observation of marked capsular lesions has been largely prevented.

There are several possible explanations for the occurrence of capsular lesions in the kidneys of guina pigs injected with neutral, or nearly neutral doses of toxin and antitoxin, but none of those thus far evolved seems very satisfactory.

1. The epithelium of Bowman's capsule may be sensitive to quantities of free diphtheria toxin too small to cause a local lesion, gross or microscopic, at the point of inoculation. This seems improbable in view of the small amount of toxin required to cause a distinct local lesion (see Table VII). Indeed, the indications are that with pure toxin no capsular lesion is produced, ordinarily, until after the local skin lesion is fairly well established.

2. The toxin-antitoxin mixture may be dissociated in vivo, as is usually assumed. In this case, the cells of the kidney, especially those of Bowman's capsule, may have a special affinity for the toxin, or else they may merely be exposed especially to the action of the toxin when it is excreted in the urine, the cells of the capsule showing the earliest injury, because they stand first in the uriniferous

system. Attempts to demonstrate dissociation of the toxin-antitoxin in vivo have thus far failed, nor has it been possible to show any decided affinity of kidney emulsions for toxin in vitro.

3. As has already been briefly suggested, a toxic fraction may be present in the diphtheria filtrate differing from the characteristically toxic fraction in that it does not cause a local oedema and necrosis at the site of inoculation, somewhat analogous to Ehrlich's "Toxon." As is indicated by the experiments recorded in preceding tables, such a substance might be present in considerable quantities in apparently neutral mixtures, though the evidence obtained in the present study suggests that it is bound by an excess of antitoxin, since capsular lesions were not observed with decidedly overneutralized mixtures. Such a toxic fraction might have a special affinity for the cells of the capsule, or again these cells might be injured first only because of their position. Though such a substance is purely hypothetical, the fact that capsular lesions may be caused by nearly neutral toxin-antitoxin mixtures, producing grossly the slightest and most fleeting local lesions, while with pure toxins they occur only after the establishment of a distinct local lesion, suggests strongly that some such substance may be present in quantity in the toxin-antitoxin mixture. As shown in Table VII, it may also be present in appreciable amounts in quantities of pure toxin, at least as small as 1/1000 to 1/5000 c.c.

4. The subcutaneous tissues, which are so very susceptible to the injurious action of diphtheria toxin, may also have, in the living animal, an affinity for the antitoxin, especially if the toxin and antitoxin have not been in contact with each other for too long a time before injection. Impregnated with antitoxin, they would probably not be susceptible to the action of the freed toxin, which might then be taken up by the lymphatics and find its way to the kidneys, through the circulation. This would explain as well as the theory of dissociation in vivo the presence of free toxin in the body after the injection of neutral, or nearly neutral, mixtures of toxin and antitoxin. The possibility of such an affinity has been vaguely suggested by some experiments on the absorption of toxin-antitoxin mixtures with tissue emulsions, but no clear-cut results were obtained.

5. Park, Famulener and Banzhaf (33), in discussing the absorption of concentrated diphtheria antitoxin (globulin) from the subcutaneous tissue, state that any such preparation tending to cause a local inflammatory reaction, with infiltration, tends also to decrease

the rate of absorption. In the case of a toxin-antitoxin mixture which, after thirty minutes' incubation at room temperature, is still capable of producing at the site of inoculation slight thickening of the skin, or a slight and fleeting oedema, it may be that, the mixture being insufficiently combined at the time of inoculation, and the passage of the antitoxin into the circulation being prevented by the presence of an inflammatory exudate, some of the toxin passes in alone and causes its injury to the internal organs before sufficient antitoxin can enter and prevent it. This theory assumes different rates of diffusion for the toxin and the globulin solution in the presence of an inflammatory exudate, a difference which might not be found to exist in practice. Nevertheless, the experiments of Martin and Cherry (27) on the filtration of toxin-antitoxin mixtures through gelatin, before they were too firmly combined, and those of Arrhenius and Madsen (1) showing that toxin diffuses ten times as rapidly as antitoxin, lend a certain amount of weight to this theory. Like the others, however, it is hypothetical and unsatisfactory, and has the added disadvantage of applying only to slightly underneutralized, or incompletely combined, mixtures of toxin and antitoxin.

Excretion of diphtheria toxin by the kidneys of guinea pigs that have received intravenous injections of (a) toxin and (b) toxin-antitoxin mixtures, both neutral and slightly toxic.

The question whether or not diphtheria toxin is excreted as such by the kidneys after injection into animals is one that has been frequently debated. Cobbett (8) observed that guinea pigs and rats injected with diphtheria toxin excreted, in the next few hours, urine which produced, when injected subcutaneously into guinea pigs, local lesions typical of this toxin, and in one instance death. Diphtheria antitoxin, when mixed with the urine, could prevent the manifestations of toxic action, while urine from normal guinea pigs gave completely negative results. Enriquez and Hallion (11) quote Bouchard as saying that diphtheria toxin is rapidly eliminated through the urine. Pettit (35) noted the presence of active toxin in the urine of rats that had received repeated sublethal doses of toxin, as well as in the urine of those that had received one large sublethal dose intravenously. In a later paper (36) he reports that if 1–2 c.c. of diphtheria toxin is injected subcutaneously into a rat, and 3–5 c.c.

of the filtered urine subsequently injected under the skin of a 250-gram guinea pig, the animal will die in 1–3 days with typical pleural and peritoneal exudates, pulmonary congestion and adrenal hemorrhage, all of which can be prevented if antitoxin is mixed with the urine. Goodman (16) speaks of the surrender of diphtheria toxin by the subcutaneous tissues of the guinea pig to the blood, and thence to the organs and to the kidneys for excretion, observing that it has been shown that the toxin is excreted in the urine a few hours after injection. Faber (12) states that diphtheria toxin does not pass through the glomerular filter into the urine, apparently basing the statement on the work of Bomstein (5), who said that toxin was not excreted into the urine. Dean (9), in commenting on the work of Cobbett, remarks that toxin appears to be excreted in the urine only when the system is flooded with it. This may be true, but at any rate it seems certain that it can be detected in the urine of guinea pigs following their intravenous injection with sufficient quantities of toxin. In the experiments to be described, only very large intravenous doses of toxin were employed, but there seems no doubt that in such cases free toxin was excreted in the urine. The objection may be raised that the toxin present in the urine came there not as a true product of excretion through the intact glomerular tuft, but merely through the rupture of some of these loops and the consequent passage of toxin-laden blood into the uriniferous tubules. Though this possibility can not be absolutely denied, it must be noted that if there was any blood present in the urine, it was too small a quantity to give it the slightest tinge. The protocol of a typical experiment is given in Table XI.

The lesions, gross and microscopic, seen in these two guinea pigs are typical of acutely fatal diphtheria intoxication, especially those in the lymphoid tissue. A section from a Malpighian corpuscle in the spleen of guinea pig 45 is shown in Plate XXVII, Fig. 1. Here may be seen in a very advanced stage the pyknosis and karyorrhexis of cell nuclei described by Oertel (30), and noted in experimental intoxications by Welch and Flexner (43, 44), Flexner (14) and others. Taken in conjunction with the autopsy findings of generalized hemorrhage and oedema, the presence of this typical lesion in the lymphoid tissue may be considered as conclusive evidence that free diphtheria toxin was present in the injected urine in quantity sufficient to kill acutely. The evidently massive dose of toxin that was injected in the 1.9 c.c. of urine suggests strongly that it must have passed

TABLE XI.

Excretion of diphtheria toxin in the urine of guinea pigs after its intravenous injection.

Guinea Pig 43. Weight, 315 grams.		
11.10.21	10.08 A.M.	2 c.c. (500 m.l.d.) of ripe diphtheria toxin in the vein.
	3.05 P.M.	Scanty urine passed; 1.9 c.c. injected subcutaneously into guinea pig 45.
11.11.21	12.15 A.M.	Breathing very slow and labored.
	6.30 A.M.	Found dead.
		Autopsy.—No marked post-mortem changes except that the colon was greatly distended with gas. Liver pale and mottled, showing large foci of necrosis. Spleen mottled with dark areas like hemorrhages. Kidneys somewhat congested. Adrenals only slightly congested. Some increase in peritoneal fluid. Lungs congested and somewhat oedematous. Sections showed massive hemorrhages and necrosis in the organs, with extreme pyknosis and karyorrhexis in the lymphoid tissue.
Guinea Pig 45. Weight, 195 grams.		
11.10.21	3.15 P.M.	1.9 c.c. of fresh urine from guinea pig 43 given subcutaneously.
11.11.21	9.00 A.M.	Very sick and weak. Little oedema at the point of inoculation.
	11.35 A.M.	Breathing shallow and labored.
	11.50 A.M.	Dead.
		Autopsy.—Slight subcutaneous oedema at the point of inoculation. Adrenals markedly hemorrhagic throughout. Kidneys somewhat congested. Lungs congested and slightly oedematous. Moderate hydrothorax. Marked increase in pericardial fluid. Sections showed massive destructive hemorrhage of the adrenals, congestion and hemorrhage of other organs and very extensive pyknosis and karyorrhexis of cell nuclei in the lymphoid tissue.

through the intact glomerular capillaries. It seems improbable that so much toxin would have been present in the urine five hours after its injection unless all the glomerular filters had been at work. It is noteworthy also that in the sections of the kidneys of guinea pig 43 the glomerular capillaries, though greatly engorged, showed little or no tendency to rupture into the capsular space.

In Table XII an experiment is recorded in which one guinea pig was protected by antitoxin against the fatal effects of urine from animals which had been injected intravenously with massive doses of diphtheria toxin, while the control, injected with a mixture of urine and normal horse serum, died.

TABLE XII.

Excretion of diphtheria toxin in the urine of guinea pigs after its intravenous injection.

Guinea Pig 49. Weight, 470 grams.			
	11.11.21	8.30 P.M.	5 c.c. (1,250 m.l.d.) of ripe diphtheria toxin in the vein.
	11.12.21	9.00 A.M.	Found dead.
			Autopsy.—Stomach and intestines slightly congested. Spleen mottled with hemorrhages. Kidneys slightly congested. Adrenals congested, especially in the medulla. Lungs congested and oedematous. 2.2 c.c. of urine removed aseptically from the bladder and injected subcutaneously into guinea pigs 51 and 52.
Guinea Pig 51. Weight, 215 grams.			
	11.12.21	10.35 A.M.	1.1 c.c. of fresh urine from guinea pig 49 plus .5 c.c. diphtheria antitoxin injected subcutaneously.
	11.13.21	9.00 A.M.	Fairly marked local oedema.
	11.14.21	3.00 P.M.	Oedema receding. Seems well.
			Note: This guinea pig remained well. There was either too much toxin present to be completely neutralized by the antitoxin, or else the oedema was due to the irritating effects of the urine.
Guinea Pig 52. Weight, 215 grams.			
	11.12.21	10.35 A.M.	1.1 c.c. of fresh urine from guinea pig 49 plus .5 c.c. normal horse serum injected subcutaneously.
	11.13.21	9.00 A.M.	Slight local oedema.
	11.14.21	3.00 P.M.	Found dead.
			Autopsy.—Oedema of the subcutaneous tissues. Adrenals hemorrhagic throughout. Lungs congested and oedematous.

TABLE XIII.

Failure of guinea pigs to excrete diphtheria toxin in the urine after intravenous injection of massive doses of very slightly underneutralized toxin-antitoxin.

Guinea Pig 176. Weight, 630 grams.

10.18.21 11.25 A.M. 10 c.c. toxin-antitoxin mixture in vein. (Mixture = 2.3 c.c. ripe toxin plus 10 c.c. antitoxin dilution, or 10 volumes of toxin slightly in excess of the L-o dose plus 10 units of antitoxin. Mixture incubated 50 minutes at room temperature before injection.)

2.40 P.M. Urinated 5 c.c. Injected subcutaneously into guinea pig 178.

10.19.21 10.20 A.M. Urinated 5 c.c. Injected subcutaneously into guinea pig 179.

10.22.21 11.25 A.M. *Killed. Autopsy.*—Nothing abnormal seen. Sections of the kidneys showed some congestion, chronic interstitial nephritis, a few casts, some albuminous material in the capsular space. There was occasional slight thickening of the cells of Bowman's capsule, but nothing at all definite.

Guinea Pig 178. Weight, 235 grams.

10.18.21 2.40 P.M. 5 c.c. of fresh urine from guinea pig 176 injected subcutaneously.

10.19.21 9.15 A.M. Fairly marked firm oedema.

10.20.21 9.00 A.M. Slight residual thickening.

10.22.21 12.00 M. *Killed. Autopsy.*—Abdominal wall still slightly thickened. No other findings. Sections of the skin and abdominal wall showed some increase in subcutaneous fibrous tissue, some oedema, hemorrhage and considerable leukocytic infiltration, all of which may have been due to the urine as such. Sections of the adrenals showed slight superficial congestion. Those of the kidneys showed some congestion but no definite capsular thickening.

TABLE XIII.—*Continued.*

Guinea Pig 179. Weight, 265 grams.

10.19.21 10.20 A.M. 5 c.c. of fresh urine from guinea pig 176 injected subcutaneously.

10.20.21 9.00 A.M. Fairly marked flabby oedema.

10.23.21 11.10 A.M. *Killed.* *Autopsy.*—Slight local thickening of the abdominal wall, chiefly along the midline. Kidneys and liver hyperemic. Adrenals very slightly congested superficially. Lungs show patchy congestion. Sections of the skin and abdominal wall showed a subcutaneous abscess in the midline. Sections of the adrenals showed very slight superficial congestion, and a few scattered cells about the medulla containing pigment resembling hemosiderin. Sections of the kidneys showed congestion, a little chronic interstitial nephritis and no thickening of Bowman's capsule.

Guinea Pig 177. Weight, 250 grams.

10.18.21 11.50 A.M. 2 c.c. subcutaneously of the same toxin-antitoxin mixture that was injected intravenously into guinea pig 176.

10.19.21 9.15 A.M. Very slight oedema at the point of inoculation.

10.20.21 9.00 A.M. Very slight residual thickening of the skin.

10.22.21 11.40 A.M. *Killed.* *Autopsy.*—Nothing abnormal noted, except for slight congestion of the left adrenal and an old pneumonic process in a small lobe of the lung. Sections of the skin and abdominal wall showed some possible increase in fibrous tissue with a few necrotic-looking leukocytes among the fibroblasts. Sections of the adrenals showed slight peripheral congestion of the left. Sections of the kidneys showed some congestion and occasional slight thickening of Bowman's capsule, but nothing at all marked or even very definite.

Some experiments were carried out in an effort to determine whether mixtures of toxin and antitoxin were dissociated after injection into the animal body. It was thought that if such dissociation could be shown to occur, it might explain the swelling and proliferation of Bowman's capsule observed in the kidneys of guinea pigs following the subcutaneous injection of neutral, or nearly neutral mixtures of toxin and antitoxin. It was hoped that if such dissociation actually took place, free toxin might perhaps be demonstrated in the urine of guinea pigs following the intravenous injection of large volumes of neutral, or nearly neutral toxin-antitoxin. These hopes have been disappointed. It has been impossible to demonstrate, in the urine of guinea pigs injected intravenously with large volumes of neutral, or nearly neutral toxin-antitoxin, any action which could with certainty be attributed to the presence of free diphtheria toxin. Moreover, the guinea pigs which received these large volumes of toxin-antitoxin intravenously failed themselves to show any definite capsular lesion. The protocol from one such experiment is given in Table XIII.

It appears from the experiment recorded in Table XIII that a very slightly under-neutralized mixture of toxin and antitoxin, when injected intravenously into guinea pigs, is not dissociated to any appreciable extent. If it is dissociated, it must be so gradually that a demonstrable amount of toxin is excreted only over a long period, unless the freed toxin is bound by other body cells before it reaches the kidney, or unless it is accompanied by some antitoxin in its passage through the glomerular capillaries. The injection into the circulation of so large a volume of fluid (10 c.c. in a 630-gram guinea pig) must be followed shortly by a compensatory excretion of fluid by the kidney, so that the first specimen of urine, taken about three hours after the injection, probably could not have been expected to contain any toxin even if a little had been freed. The point may be raised that in this experiment specimens of urine were obtained only at 3 and at 23 hours after the injection, while if a specimen had been obtained at some time between those two, it might have been found to contain free toxin. While the objection must be allowed for this particular experiment, similar experiments in which specimens of urine were obtained during this same period failed to show any more positive evidence of the presence of free toxin in the urine. It would seem that if actual dissociation of toxin and antitoxin takes place in vivo, as is commonly supposed, some better method of detecting it must be devised.

The impression has been gained that toxins lose their ability to cause capsular lesions in neutral, or nearly neutral mixtures as they age, retaining it almost undiminished up to about six months. The toxin employed in the experiments recorded in Tables XII and XIII was the same one used in most of the previous experiments, but at the time that these two experiments were performed it was some 13 months old, and no longer caused capsular lesions in the kidney in neutral mixtures as it had caused them 7 to 9 months previously, when it was only 4 to 6 months old (see Tables IV, V, VII and VIII). This may explain why little or no indication of capsular injury was seen in guinea pigs injected intravenously with nearly neutral doses of antitoxin and aged toxin, even though such massive doses were given. The freshly prepared toxin used in Table X, when mixed with antitoxin in such proportion that it caused only a slight local lesion when given in 2 c.c. quantities subcutaneously, was borne so ill when injected in multiple doses intravenously that it was almost impossible to repeat the experiment with it.

Absorption of diphtheria toxin in vitro by emulsions of various tissues from (a) guinea pig and (b) rat.

The absorption of tetanus toxin in vitro by the emulsified brains of susceptible animals, and the failure of brain emulsions derived from insusceptible animals to absorb the toxin or to protect animals against it is well known. Wassermann and Takaki (42), Marie (26), Metchnikoff (28), Besredka (4) and others have worked on this problem. I have found so far very little in the literature on the absorption of diphtheria toxin in vitro or in vivo by the organs of susceptible animals, though there are some papers on its absorption by the tissues of rats. Roux and Yersin (39) stated that the filtered extract of the spleen of a child dead of diphtheria, as well as filtered urine from one at the time of greatest prostration, killed guinea pigs and rabbits when given intravenously, but since no mention is made of the autopsy findings in these animals, and since even normal human urine and organ extracts might well be toxic for such animals when given intravenously, the assumption that diphtheria toxin was present in the injected material seems to lack proof. Moreover the toxin, if present in the splenic extract, might easily owe its presence to the blood contained in the organ. Goodman (16) observed that emulsions of spleen, myocardium, liver and brain from the rat were unable, in vitro, to bind even 2 m.l.d. of toxin sufficiently to protect a guinea pig, the effect being the same whether the tissue-toxin mixtures were kept at 20° C. or at 37° C. for thirty minutes before injection. Kuprianow (21) showed that fresh emulsions of spleen, kidney, lung and heart from the rat, when injected into guinea pigs, did not influence the action of living diphtheria cultures. Wadsworth and Vories (41) note that guinea pig brain tissue and guinea pig and dog leukocytes have no action on diphtheria toxin.

In order to test out some of the theories already elaborated regarding the special affinity of certain tissues (notably the kidney and the subcutaneous connective tissue) for diphtheria toxin, some experiments were carried out to determine, if possible, whether there was any absorbing power in the tissues of the susceptible guinea pig, demonstrable in vitro, how it differed in different tissues and how these tissues compared in their ability to absorb diphtheria toxin with the tissues of the relatively insusceptible rat. A number of experiments were run. The animals were exsanguinated, as far as possible, when well under the effect of ether, the tissues removed aseptically, ground in a sterile mortar and weighed amounts placed in small centrifuge tubes. Subcutaneous connective tissue, kidney and brain were used. The brain emulsified best, the kidney next best, the subcutaneous tissue not at all. To one or two grams of the tissue emulsions was added 2 m.l.d. of ripe diphtheria toxin plus enough sterile salt solution to bring the known fluid volume up to 3 or 4 c.c. The tissue emulsions and the fluid were then well mixed with a small pipette and the tubes allowed to stand in the icebox for about 48 hours, when they were centrifugalized at high speed and the supernatant fluid poured off and injected subcutaneously into guinea pigs. The greatest amount of fluid was always absorbed by the guinea pig subcutaneous tissue, so that, in order not to reduce too far the volume of all the injections, that from the guinea pig subcutaneous tissue was allowed to remain the smallest. This, however, did not seem to affect the outcome. Control experiments were run with a quantity of animal charcoal equal in weight to the tissue emulsions, and with toxin alone in an equal volume of salt solution, allowed to stand for the same length of time in the icebox.

It was found that, far from absorbing the toxin, all the tissue emulsions, whether from the rat or the guinea pig, seemed actually to hasten death in guinea pigs when the supernatant fluid from such toxin-tissue mixtures was injected into them. The animal charcoal never failed to remove practically all the toxin from the fluid, while toxin alone, set up for the same length of time in the icebox in an equal volume of saline, usually required a much longer period to kill than did the supernatant fluid from tissue emulsions, sometimes deteriorating to such an extent that it produced very little local effect, and caused death, if at all, only after a relatively long period (10 days). The protocol from a typical experiment is given in Table XIV.

TABLE XIV.

The effect of the subcutaneous injection into guinea pigs of the supernatant fluid from emulsions of rat and guinea pig tissues plus 2 m.l.d. of diphtheria toxin, made up to 3 c.c. with salt solution and incubated together in the icebox for 42 hours before injection.

One gram of each tissue and of animal charcoal was used. The toxin control received 2 c.c. of a toxin-saline mixture consisting of 2 m.l.d. of toxin in a 3 c.c. volume of saline, also allowed to stand 42 hours in the icebox before injection.

Guinea pig.	Initial weight in grams.	Supernatant fluid injected, c.c.	Absorbing emulsion and toxin control.	Gross skin lesion 48 hours.	Hours lived.	Last weight recorded.
261...	260	2.00	Guinea pig, brain.	Very massive.	70	....
262...	250	2.00	Guinea pig, kidney.	Fairly massive.	120	220 grams—72 hours, a loss of 12 per cent.
263...	230	1.75	Guinea pig, subcutaneous tissue.	Marked.	70	....
264...	240	2.00	Rat, brain.	Fairly massive.	70	....
265...	240	2.00	Rat, kidney.	Fairly massive.	75	205 grams — 72 hours, over 14 per cent loss.
266...	220	2.00	Rat, subcutaneous tissue.	Very marked.	70	....
267...	215	2.00	Animal charcoal.	Slight thickening.	—	235 grams — 72 hours, over 9 per cent. gain.
268...	200	2.00	Toxin control.	Marked.	124	190 grams — 72 hours, a loss of 5 per cent.

It will be seen from Table XIV that the known fluid volume of toxin and saline in which the tissue emulsions were suspended was 3 c.c. If this same volume could have been recovered, supposing none of the toxin had been absorbed by the tissue, there could not have been more than 2 m.l.d. of toxin contained in it, and probably not so much, since some further dilution with tissue fluids must have occurred. The quantity of supernatant fluid injected (2 c.c.), representing, as it does, 2/3 of the total known fluid volume of the mixture, represents also approximately 2/3 of 2 m.l.d. of toxin, or about 1.33 m.l.d. One gram of tissue emulsion was therefore unable to absorb slightly more than 1/3 m.l.d. of toxin. In the case of the guinea pig subcutaneous tissue, where only 1.75 c.c. of supernatant fluid was recovered for injection, the total volume injected represents only about 1.16 m.l.d. of toxin, and yet this guinea pig died in 70 hours, whereas the smaller control animal, injected with a volume of fluid known to have contained, originally, 1.33 m.l.d. of toxin, survived for 124 hours. Similar experiments done with 2 grams of tissue emulsion gave essentially the same results, 2 grams of tissue failing to protect against slightly more than .25 m.l.d. of toxin. A set of guinea pigs each injected subcutaneously with 2/3 of the supernatant fluid from 1.5 grams of similar tissue emulsions plus 2 m.l.d. of toxin, incubated together in the water bath 30 minutes before centrifugalization, all died at about the same time (36–48 hours), including the toxin control, except the guinea pig receiving the supernatant fluid from the mixture of toxin and animal charcoal, which remained normal. Apparently, then, the time factor is concerned in the relatively heightened toxicity of the supernatant fluid recovered from mixtures of tissue emulsion and toxin, as well as in the deterioration of toxin diluted with saline.

In his study of the resistance of rats to diphtheria toxin, Cobbett (7) observed that the serum of these animals in doses of 1 c.c. not only failed to protect guinea pigs against quantities of toxin little more than 1 m.l.d., but seemed sometimes to actually shorten their life, although the serum did not appear to be in itself toxic for guinea pigs, since no immediate ill effects followed its injection. Leuchs (22), working with botulinus toxin, found that its potency could be increased by adding to it an equal quantity of whole horse serum, or its alcohol-soluble fraction, or an emulsion of lecithin. In Table XIV it will be seen that the animals receiving supernatant fluid from guinea pig and rat kidney emulsions and toxin outlived the others by several hours, a fact which has been noted before in the case of the guinea pig kidney. This may indicate either a higher resistance on the part of these particular guinea pigs, or a slightly greater affinity on the part of the toxin for the kidney tissue, especially that of the guinea pig, than for brain or subcutaneous tissue. The difference, however, is too slight to mean very much, and has not been observed invariably. If it could be demonstrated constantly that kidney emulsions had a greater affinity, in vitro, for diphtheria toxin than had emulsions of other tissues, it would lend weight to the hypothesis that toxin, present in the circulating blood, has a special affinity for kidney tissue, and might help to explain lesions appearing in these organs before they are observed elsewhere. But since the volume of tissue in Bowman's capsule, relative to the volume of all the other types of tissue in the kidney, is so small, the affinity of this particular tissue for the toxin would have to be enormous in order to cause kidney emulsions to show distinctly greater absorbing power for the toxin in vitro than did emulsions of other organs. It seems more reasonable to assume that lesions appear first in Bowman's capsule simply because of its position at the very beginning of the uriniferous system. and that they are caused by the toxin, or some part of it, excreted in the urine.

One control which should have been run with the absorption experiments recorded above may be mentioned. It would have been well to inject another guinea pig with a toxin-saline mixture identical with that used in the toxin control recorded in the above series, the mixture differing only in the fact that it was prepared immediately before injection. This should indicate how much the toxin had deteriorated in pure saline, and whether the tissue merely failed to absorb the toxin, while at the same time it provided a medium in which toxin tended to deteriorate more slowly than in saline.

Discussion.

It is generally assumed that the neutral, or nearly neutral mixtures of diphtheria toxin and antitoxin used for immunization are gradually dissociated in vivo, the toxin molecules thus freed being taken up by the receptors of susceptible cells and so giving rise to the active production of antitoxin. Karsner and Ecker (19), in their recent book on "The Principles of Immunology," state that "the active immunization of man by the use of neutralized mixtures of toxin and antitoxin appears to provide conditions whereby dissociation progresses gradually, and the toxin is liberated in such small amounts that it does no harm and yet induces in the body antitoxin formation'' (page 24). Even more recently has appeared a paper on this subject by Opitz (31). He advocates the use of over-neutralized mixtures of toxin and antitoxin for purposes of immunization. He does not believe, with Loewenstein, that the toxin is freed through gradual decomposition of the antitoxin, but considers rather that there is a rapid dissociation, in which perhaps favorable conditions for the resorption of the smaller toxin molecule play a part. He notes also that there may be a greater avidity of the toxin for the tissue receptors than for the free antitoxin receptors. In support of his assumption that mixtures of toxin and antitoxin are dissociated in vivo he cites the following evidence:

1. An immunizing mixture of Behring's, which had become neutral through toxin deterioriation, was still able to produce antibodies on injection into animals.

2. Pure toxin dilutions whose concentration was adapted to individual susceptibility, but which contained at least 20 L-n doses, were about as effective as neutral mixtures.

3. The minimal immunizing dose must be greater than 1 L-n dose, by L-n dose being understood that quantity of toxin which, on intracutaneous injection into guinea pigs, just causes necrosis at the point of inoculation (Opitz).

Although the dissociation of diphtheria toxin and antitoxin in vivo is accepted as a fact, no experimental evidence has been presented, so far as I am aware, which definitely proves it. In the present series of experiments, where it was hoped that advantage might be taken of the fact that diphtheria toxin is excreted in the urine, no demonstrable toxin was found in the urine of guinea pigs following the intravenous injection of neutral, or nearly neutral toxin-antitoxin mixtures. It is possible that some toxin might be detected in such urine if it could all be collected over a longer period and concentrated, but it is probable that the already irritating qualities of normally concentrated guinea pig urine would be considerably enhanced by this procedure, while the toxin, if present, would almost certainly suffer marked deterioriation.

Faber (12), in his study of the effect of diphtheria toxin on the kidneys of 1,000–1,500-gram rabbits, notes that ''anatomically the secondary nature of the epithelial lesions is indicated by the fact that with small doses of toxin glomerular lesions are recognizable before tubular degeneration appears, and that with doses of medium size the glomerular lesions are much severer than the tubular. In short, endothelial injury is predominant.'' He found that, in rabbits, the maximum effect was obtained with doses of .008 c.c. per kilogram of a toxin whose m.l.d. was .005 c.c. Starting with the assumption that diphtheria toxin does not pass through the glomerular capillaries into the urine, he argued that, since water is lost from these capillaries, the toxin should become more concentrated at this point than elsewhere, and therefore should exert its most marked injury upon the capillary endothelium. Pearce and Eisenbrey (34), on the other hand, state that ''diphtheria toxin produces a nephritis which in its late stage is of the vascular type, but in its early stage is distinctly tubular.'' The latter authors worked with dogs, but their interpretation seems to me the more correct one as applied also to guinea pigs. In the present series of experiments distinct indications of injury to the glomerular loops, other than the fibrin plugs sometimes seen in the glomerular capillaries of guinea pigs injected with pure toxin and killed early, were usually only encountered late in severe intoxications, and were absent in animals injected with neutral, or nearly neutral toxin-antitoxin mixtures capable of causing marked swelling and proliferation of Bowman's capsule, which may, I suppose, be properly considered part of the tubular system.

It seems probable that the swelling and proliferation of Bowman's capsule observed in the kidneys of guinea pigs following the injection of diphtheria toxin or of neutral, or nearly neutral mixtures of toxin and antitoxin, is due to the passage through the glomerular capillaries of the whole toxin or some fraction of it. This is indicated both by the appearance of lesions in Bowman's capsule following the injection of weak toxin-antitoxin mixtures which can not with certainty be said to produce similar effects in the convoluted and collecting tubules, and by the fact that even where there has been pronounced tubular injury, as indicated by the proliferative process present, such proliferation rarely extends to the collecting tubules of the pyramid, but is confined almost entirely to the kidney cortex. Although it must be noted that the more highly differentiated cells of the capsule and of the convoluted tubules are also more susceptible to the action of injurious agents, the findings recorded above would seem to indicate that the toxic substance may be exhausted in the course of its passage through the tubules of the cortex, so that, except when the doses are large or the passage very rapid, little or none remains in the urine to injure the cells of the collecting tubules in the medulla. A disturbing factor is encountered in the observation that there may be a very extensive thickening of Bowman's capsule, with proliferation, accompanied by a most active proliferative process in the collecting tubules throughout the kidney, while at the same time there is far less proliferation, or histological evidence of injury, in the convoluted tubules (see Table X). It may be that in such cases the extensive proliferation observed in the collecting tubules has its origin in some unknown and unrelated cause, or again, the convoluted tubules may have been so injured physiologically that they are unable to react and proliferate, or they may even normally lack the power of easy regeneration possessed by the cells of the collecting tubules. In any case, it seems probable that the substance, or substances, which cause the swelling and proliferation seen in the cells of Bowman's capsule are borne by the excreted urine, not by the blood.

It seems to me that the possibility that the swelling and proliferation of Bowman's capsule is due to some fraction of the diphtheria filtrate other than the characteristically toxic one should not be overlooked. While it has not been possible to prove that the capsular lesions caused by the injection of neutral, or nearly neutral, mixtures of toxin and antitoxin are not due to free toxin which has become dissociated in vivo from its union with antitoxin, and while the view that a gradual dissociation takes place in vivo is favored by the fact that capsular lesions persist for at least four weeks after the injection of a single under-neutralized dose of toxin-antitoxin, it is at least suggestive that such lesions are caused by mixtures of toxin and antitoxin which produce no injury, gross or microscopic, at the point of inoculation in the subcutaneous tissue, while pure toxin, injected subcutaneously, must cause a fairly marked lesion at the point of inoculation before swelling and proliferation of Bowman's capsule can be detected. Although it has not been shown thus far that absorption of the toxic broth in vitro with emulsions of guinea pig or rat tissues removes the substance present in it which has the power to cause these capsular lesions, it is possible that more exhaustive absorption might produce the desired effect. Further work on this question is planned.

Exactly what is involved in the marked swelling of the capsules is not known. It may be due to a simple oedema of the cells, analogous to the oedema produced in most of the tissues of the body by diphtheria toxin. It is a little hard, however, to understand why a simple oedema of these cells should persist for four weeks or more, without further injections of toxin and its excretion through the glomerular tufts, unless it is true that the toxin-antitoxin mixtures injected in these cases remained in the body throughout that time and were gradually dissociated. It is also difficult to understand why, if the swollen appearance of the capsular cells is due to the action of whole diphtheria toxin, there is not more indication of necrosis of these cells, such as is commonly found in other cells of the body similarly affected. Apparently actual necrosis of these swollen cells is by no means the rule, especially in the kidneys of guinea pigs that have received neutral, or nearly neutral toxin-antitoxin mixtures, while desquamation of such cells is rarely observed.

It seems rather curious that there should have been so little study of the pathological effects of mixtures of diphtheria toxin and antitoxin lying between the L-o and the L-+ doses. I have seen only one reference to the microscopic examination of guinea pig tissues, other than nervous tissue, following the injection of toxin-antitoxin mixtures. This is contained in a short paper by Frothingham (15) on the production of a glomerular and arterial lesion in rabbits' kidneys by the injection of diphtheria toxin, and he merely mentions in passing that two guinea pigs, injected with toxin-antitoxin mixtures to produce paralysis, survived a month and showed, at autopsy, marked fatty degeneration in many organs, but no morphological lesion or fatty degeneration in the vessels. Other papers, such as that by Dreyer and Madsen (10), comment, if at all, only on the gross pathology. While the work done in the present study suggests that toxin-antitoxin mixtures prepared with well-ripened toxins produce no lesion in Bowman's capsule, following the injection into guinea pigs of mixtures of graded toxicity, until one gets a toxic effect at the site of inoculation (slight persisting oedema, and perhaps some necrosis), the toxin-antitoxin mixtures used in this country for the immunization of children are slightly under-neutralized and produce in guinea pigs, when given subcutaneously in 2 and 3 c.c. quantities, a definite capsular swelling and proliferation. It may be that this effect upon the capsule is of no importance. While it appears to persist in guinea pigs for some weeks after the injection of decidedly under-neutralized toxin-antitoxin mixtures, it has not been observed, in this time, to lead to scarring of the capsule or obliteration of the capsular space. It is hoped that further work may be done on the physiological effects of this capsular thickening, possibly in some larger animals, in which it would be easier to do careful urinalyses. The work of Karsner and Denis (18) suggests that rabbits might be suitable for this purpose.

SUMMARY.

1. A brief account is given of some lesions frequently observed in the tissues of supposedly normal guinea pigs, including chronic interstitial nephritis, tubular nephritis (as indicated by the proliferative process observed in the cells of the convoluted and collecting tubules), and widespread infiltration of the connective tissue everywhere with great masses of eosinophilic cells. Though all these things may occur independently, they are frequently seen in combination in the same animal, and it is thought that they may all owe their presence to some relatively mild chronic intoxication.

2. An account is given of the chief lesions hitherto described as characterizing experimental diphtheria intoxications, and an effort is made to correlate the more important lesions as observed in the guinea pig with regard to the time required for their development. The results are tabulated.

3. A lesion of Bowman's capsule is described, consisting of swelling and proliferation of the capsular cells, which in well-marked cases assume almost the appearance of cuboidal epithelium, and in sections properly stained with eosin and methylene blue are distinctly basophilic. The lesion does not appear until about the fourth day after subcutaneous injection of (a) quantities of pure toxin capable of causing a distinct local lesion when given subcutaneously, especially those lethal on or shortly after the fourth day, and (b) mixtures of toxin and antitoxin all the way from the L-o to the L-+ dose. Marked and widespread thickening of the capsules, with proliferation, has been observed as late as four weeks after subcutaneous injection of considerably under-neutralized mixtures of toxin and antitoxin, but no scarring of the capsule or obliteration of the capsular space has been seen. The ultimate fate of the capsular lesion is still unknown, but it is thought to subside gradually and leave no trace. Some tentative theories are advanced to account for the pathological effects of neutral, or nearly neutral, mixtures of toxin and antitoxin. It is thought that toxins gradually lose the property of causing capsular lesions in neutral, or nearly neutral, mixtures as they age, retaining it for about six months. Two toxins

are compared for their ability to cause lesions in the capsule of Bowman, both in a pure state and when neutralized with antitoxin. The curious fact was elicited that the less potent toxin was able to cause both local skin lesions and capsular lesions in a considerably higher dilution than was the more potent one, while at the same time its toxon fraction was much larger than was that of the more potent toxin. These facts seem to lend weight to the theory that the capsular lesion is caused by some fraction of the toxin other than the characteristically toxic one, and that it may be associated with the toxon content of the filtrate.

4. An account is given of the capsular lesions and the fatal outcome in parallel series of guinea pigs injected (a) subcutaneously and (b) intravenously with doses of toxin-antitoxin lying between the L-o and the L-+ mixtures. It was found not only that death occurred at a point of lower toxicity in the series receiving the intravenous injections, but that the least toxic mixture capable of causing a lesion in Bowman's capsule produced a more marked effect in the intravenous series than in the series injected subcutaneously.

5. Some unsuccessful experiments are recorded in which an attempt was made to detect free toxin in the urine of guinea pigs injected intravenously with large volumes of neutral, or nearly neutral, toxin-antitoxin. These experiments were performed in an effort to demonstrate in vivo the dissociation of toxin and antitoxin which is said to occur following the injection of such mixtures during the process of immunization.

6. Unsuccessful experiments are recorded in the absorption of diphtheria toxin in vitro by emulsions of guinea pig and rat brain, kidney and subcutaneous tissue. One gram of such an emulsion, allowed to stand about 48 hours in the icebox in contact with 2 m.l.d. of toxin contained in a 3 c.c. volume of salt solution, not only failed to absorb any of the toxin but appeared to actually enhance the toxicity of the supernatant fluid recovered from the mixture, when this was injected subcutaneously into guinea pigs.

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EXPLANATION OF PLATES.

PLATE XXII.

FIG. 1. Chronic interstitial nephritis from the kidney of "normal" guinea pig 5. × 96.

FIG. 2. Chronic interstitial nephritis from the kidney of "normal" guinea pig 5. A small detail taken from Figure 1. × 416.

PLATE XXIII.

FIG. 1. Infiltration of the subcutaneous and intermuscular connective tissue of "normal" guinea pig 4 with great masses of eosinophilic cells. A clump is shown in the loose connective tissue about a blood vessel in the abdominal wall. × 416.

FIG. 2. Thickening and proliferation of Bowman's capsule in the kidney of a guinea pig dying on the sixth day after subcutaneous injection of ⅝ the m.l.d. of diphtheria toxin. A glomerulus is shown, with its capsule containing at (*A*) a large cell in mitosis. Evidences of prolonged toxin action are present in the tubular casts seen at (*B*) and (*C*). × 416.

PLATE XXIV.

FIG. 1. Massive skin lesion in guinea pig 92, killed on the 4th day after subcutaneous injection of a large dose of diphtheria toxin. The epidermis is completely necrotic, and shows hemorrhages and diffuse leukocytic infiltration. There is massive oedema of the subcutaneous connective tissue, sparsely infiltrated with leukocytes. The underlying muscle is oedematous, somewhat disorganized and infiltrated with leukocytes. × 19.

FIG. 2. Thickening and proliferation of Bowman's capsule in the kidney of guinea pig 92. Mitoses in the capsular cells may be seen at (*A*) and (*B*). This picture gives an excellent idea of the appearance of Bowman's capsule when the lesion is marked. × 416.

PLATE XXV.

FIG. 1. Section through the skin and abdominal wall of guinea pig 145, killed four days after subcutaneous injection of the L-o mixture of diphtheria toxin and antitoxin. The skin, subcutaneous tissue and underlying muscle are absolutely normal in appearance. × 19.

FIG. 2. Thickening and proliferation of Bowman's capsule in the kidney of guinea pig 145. At (*A*) is a large clear cell in mitosis. × 416.

PLATE XXVI.

FIG. 1. Section through the skin and abdominal wall of guinea pig 146, killed four days after subcutaneous injection of a mixture of diphtheria toxin and antitoxin so little in excess of the L-o dose that it caused only the slightest and most fleeting thickening of the skin. The sections showed nothing which might be considered due to the action of the toxin except occasional slight extravasations of blood just under the epidermis and some congestion of the small subcutaneous blood vessels. × 33.

FIG. 2. A group of glomeruli from the kidney of guinea pig 146, each one of which shows a distinctly thickened capsule. × 126.

PLATE XXVII.

FIG. 1. Section from the spleen of guinea pig 45, dead 20.5 hours after subcutaneous injection of 1.9 c.c. of fresh urine from a guinea pig which had received, five hours previously, a large intravenous dose of diphtheria toxin. The picture shows a Malpighian corpuscle, in which is present to a very marked degree the pyknosis and karyorrhexs of cell nuclei described by Oertel in human diphtheria, and by Welch and Flexner, Flexner and others in experimental diphtheria intoxications. × 416.

1-86

PLATE XXII.

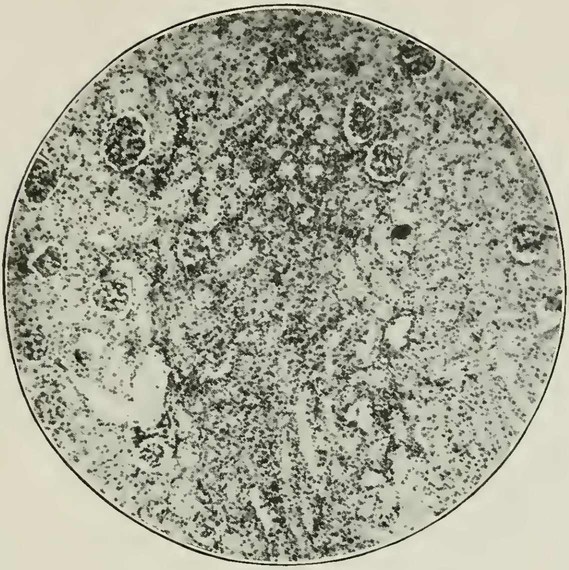

FIGURE 1.

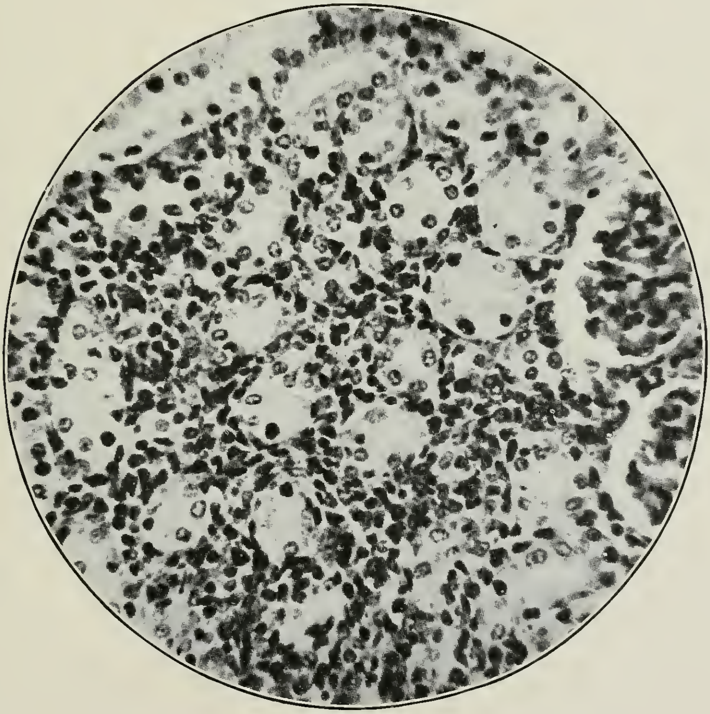

FIGURE 2.

.566

PLATE XXIII.

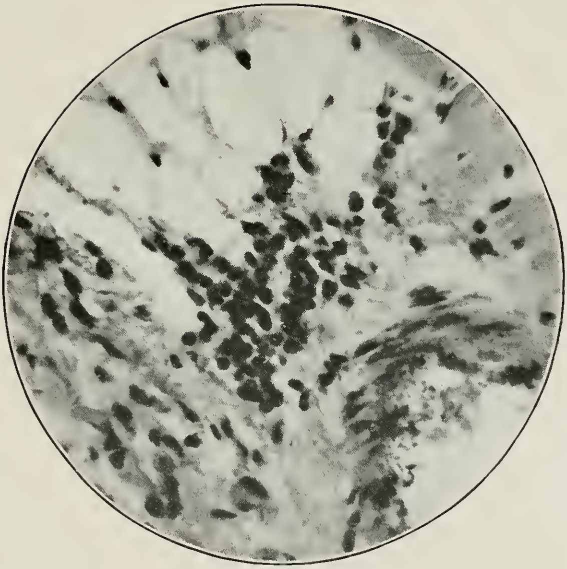

FIGURE 1.

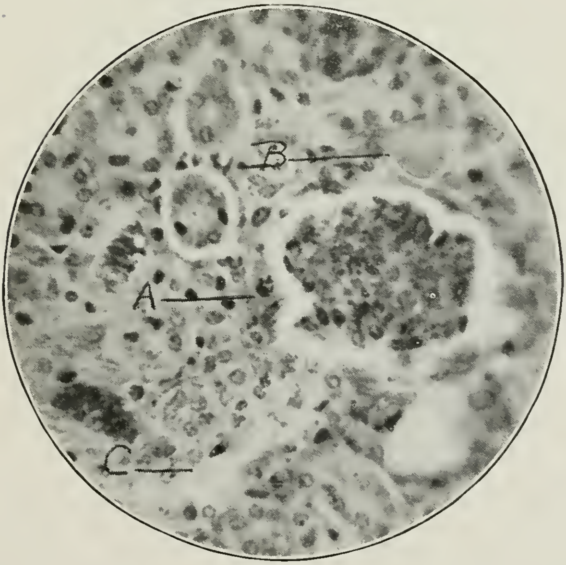

FIGURE 2.

PLATE XXIV.

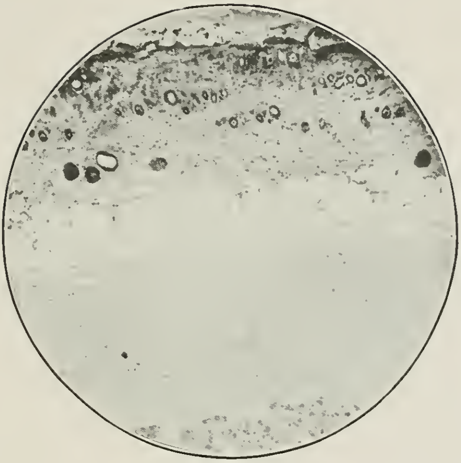

FIGURE 1.

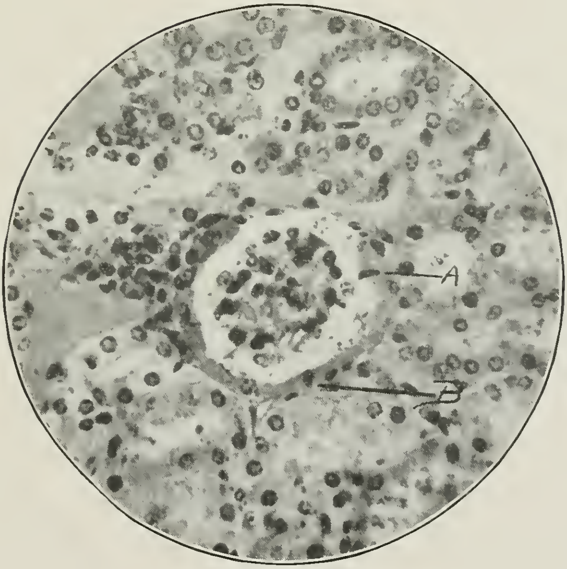

FIGURE 2.

PLATE XXV.

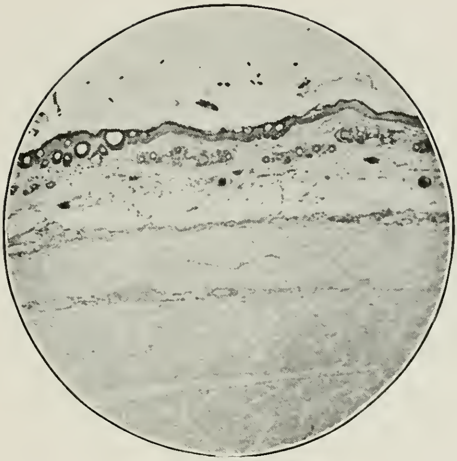

Figure 1.

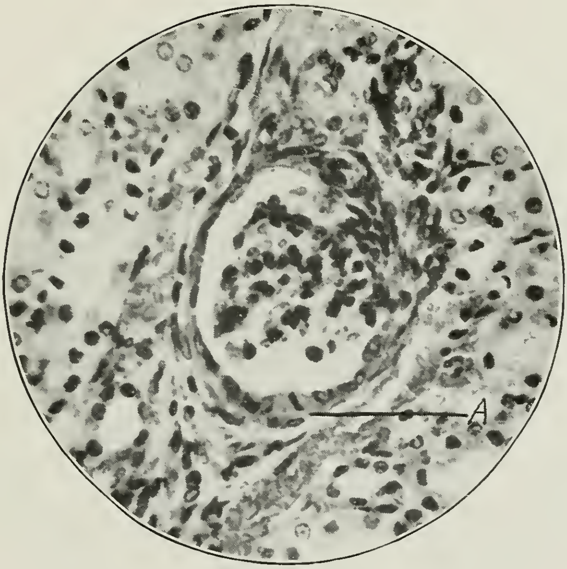

Figure 2.

5865

PLATE XXVI.

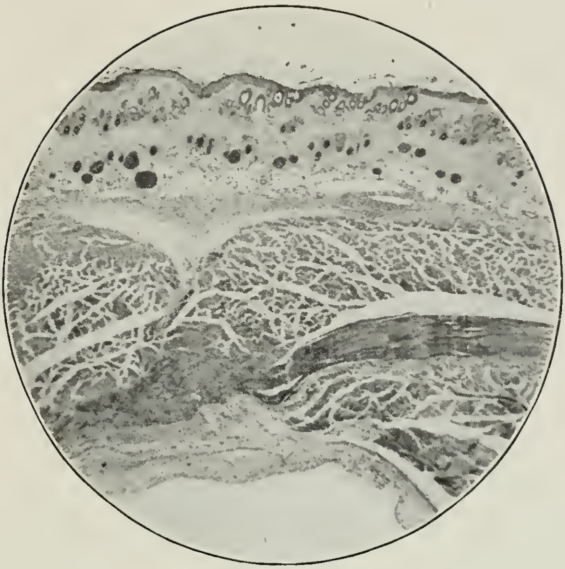

Figure 1.

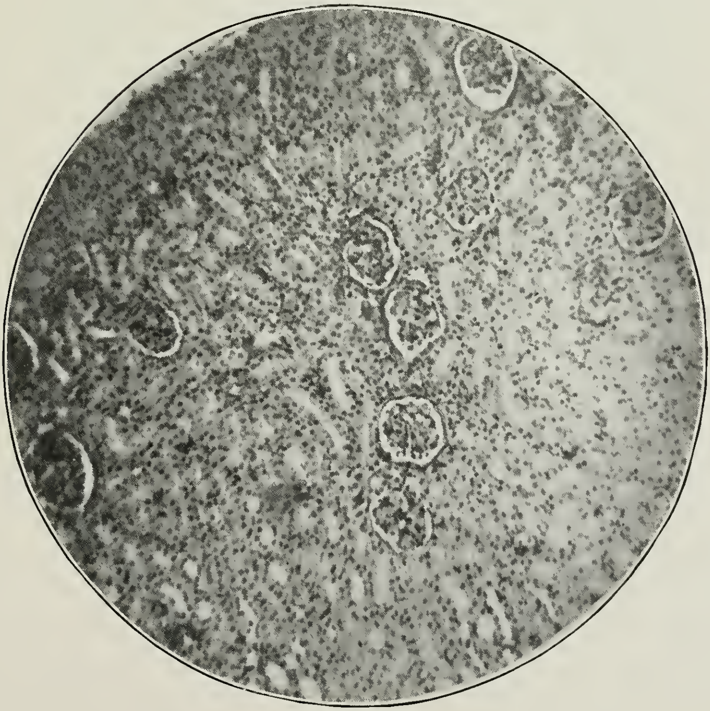

Figure 2.

PLATE XXVII.

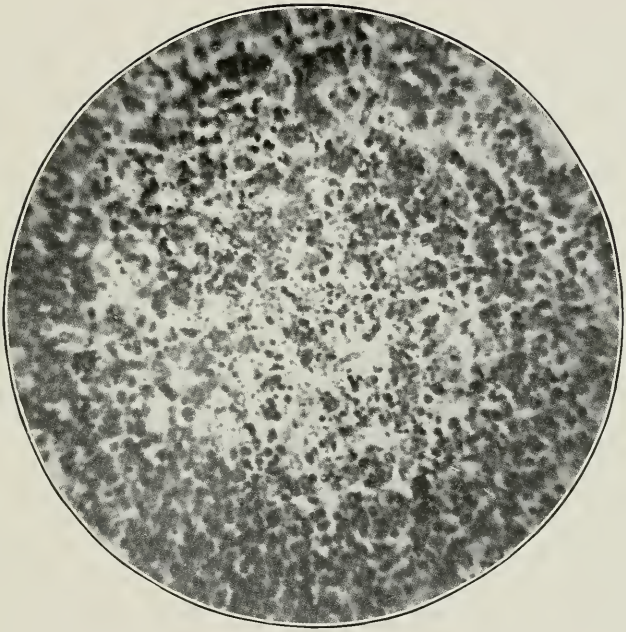

FIGURE 1.

PRELIMINARY DISCUSSION OF THE CORRELATION BETWEEN ILLITERACY AND MORTALITY IN AMERICAN CITIES.*

By RAYMOND PEARL and MORRILL L. ILSLEY.

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I. Introduction.

In a recent review of the vital statistics of this country Pearl (1921) was impressed by the thought that one of the greatest difficulties with which the health officer has to contend, in his efforts to ameliorate the conditions which make for morbidity and mortality, must surely be the ignorance of great masses of the population with which he has to deal. So much of effective public health work goes back, upon final analysis, to a basis of personal hygiene, that the dense ignorance and, what is worse, sheer stupidity of a not negligible fraction of our population must be a limiting factor. No attempt, however, so far as we are aware, has ever been made to evaluate statistically this relation. It has seemed desirable to undertake, in a preliminary way, the examination of certain data bearing upon the matter.

The attempt to measure intelligence precisely is a rather new departure in psychology, and none of the results of such endeavors may as yet be safely transferred from the special classes upon which they have been made to the general population. We are lacking, perhaps fortunately, any precise general statistics as to the average intelligence (or stupidity), or the distribution of grades of intelligence, in the population as a whole. The nearest approach to such data is found in the statistics of illiteracy.

This approach is obviously not close. An illiterate person may be, and not seldom is, naturally rather above the average in intelligence. Conversely, many literate persons are very feeble intellectually. But, with all due regard to these trite facts, it remains undeniable that, in a broad statistical sense, the illiteracy percentage of a community is a rough index of the extent to which that community, as a group or population, falls short in its average equipment for meeting the battle of existence. In the complex and highly integrated social organization of present-day man in urban population aggregates, ability to read and write is a major asset in maintaining that general awareness of one's surroundings which is so vital an element in the struggle for existence. Those persons who lack this ability are unquestionably, on the average, of a rather low grade of general intelligence, even though there may be occasional brilliant exceptions. Furthermore it cannot be denied that the illiterate person is *ignorant,* as compared with the literate, on the average.

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* Papers from the Department of Biometry and Vital Statistics, School of Hygiene and Public Health, Johns Hopkins University. No. 62.

There are available, in the reports of the Census Bureau, data as to the number of illiterates and their proportion to the total population in all significant demographic units. Recognizing fully that percentage of illiteracy is not a precise index of the average ignorance of a population, we have nevertheless thought it worth while to examine the correlation of this variable with mortality.

The following general data on illiteracy in the United States, as revealed in the 1910 and earlier census counts, are of interest for purposes of orientation. (*Cf.* Talbot, W., 1916.) Roughly, five and a half million people in this country were illiterate in 1910. Of these, over a million could speak no English. The census divided the illiterates into four classes: native white (1,534,272), foreign born (1,650,361), negro (2,227,731), others (103,859). The negro illiterates decreased from 3,150,000 in 1880 to 2,227,731 in 1910, native born white illiterates decreased from 2,255,460 to 1,534,272, and after 1890 there was a decrease of illiteracy among native born of native parents from 1,890,723 to 1,378,884 in 1910, while among native born of foreign or mixed parentage the change was from 174,280 to 155,388.

The number of illiterates steadily increased (up to 1910 at least) in Massachusetts, Rhode Island, Connecticut, New York, New Jersey, Pennsylvania, Illinois, North Dakota, Nebraska, Montana, Wyoming, Colorado, New Mexico, Arizona, Utah, Nevada, Washington, California. The greatest increase was in the New England and Middle Atlantic States, there having been an increase of approximately 140 per cent. in twenty years in these states, while on the other hand there was a decrease of thirty per cent. in the same period in the South Atlantic States.

The percentage of illiteracy in the United States was lowered

TABLE I.

Percentage of illiteracy among native whites, the foreign born, and negroes at certain periods. Census data.

Class.	1880.	1890.	1900.	1910.
Native white				
Per cent. of total population	73.5	73.0	74.5	74.4
Per cent. of illiteracy	9.4	6.2	4.6	3.0
Foreign born				
Per cent. of total population	13.1	14.5	13.4	14.5
Per cent. of illiteracy	12.0	13.5	12.9	12.7
Negroes				
Per cent. of total population	13.1	11.9	11.6	10.7
Per cent. of illiteracy	68.0	57.1	44.5	30.4

during twenty years from 13.3 per cent. in 1890 to 10.7 in 1900 and 7.7 in 1910; but the number of foreign born illiterates increased 43 per cent. in the same pediod.

More than two thirds of all illiterates were in 1910 country dwellers (3,748,031), of whom 1,342,372 were native whites, 477,870 foreign born, 1,834,458 negroes. The illiterates in cities were divided thus: 191,900 native whites, 1,172,491 foreign born, 393,273 negroes. Therefore the rural illiteracy problem lies with the negroes and native whites, and the urban with the foreign born. Foreign born illiterates are mainly in towns and cities of the New England States (222,030), Middle Atlantic States (582,756), East North Central States (217,771); native white illiterates mainly in rural communities of the South Atlantic States (400,507), East South

TABLE II.

Age distribution of illiterates in 1910.

Age.	Number.	Per cent.	White (per cent.).	Negro (per cent.).
10–14	370,136	6.7	1.8	18.9
15–19	448,414	9.0	2.8	20.3
20–24	622,073	11.2	4.6	23.9
25–34	1,102,384	19.9	5.2	24.4
35–44	940,510	17.0	5.4	27.7
45–54	829,153	15.0		
55–64	607,754	11.0	6.7	52.7
65	573,799	10.4	9.4	74.5

Central States (362,178), West South Central States (217,719); and negroes, who are illiterate, mainly in rural communities of the South Atlantic States (810,526), East South Central States (581,250), West South Central States (411,370).

The age distribution of the illiterate population in 1910 was as shown in Table II.

Table III shows the distribution of illiteracy by states in 1910.

TABLE III.

Percentage of illiteracy by states.

Iowa	1.7	New York	5.5
Nebraska	1.9	New Jersey	5.6
Oregon	1.9	Oklahoma	5.6
Washington	2.0	Pennsylvania	5.9
Idaho	2.2	Connecticut	6.0
Kansas	2.2	Nevada	6.7
Utah	2.5	Maryland	7.2
South Dakota	2.9	Rhode Island	7.7
Minnesota	3.0	Delaware	8.1
Indiana	3.1	West Virginia	8.3
North Dakota	3.1	Texas	9.9
Michigan	3.2	Kentucky	12.1
Ohio	3.2	Arkansas	12.6
Wisconsin	3.2	Tennessee	13.6
Wyoming	3.3	Florida	13.8
California	3.7	Virginia	15.2
Colorado	3.7	North Carolina	18.5
Illinois	3.7	New Mexico	20.2
Vermont	3.7	Georgia	20.7
Maine	4.1	Arizona	20.9
Missouri	4.3	Mississippi	22.4
New Hampshire	4.6	Alabama	22.9
Montana	4.8	South Carolina	25.7
Dist. of Columbia	4.9	Louisiana	29.0
Massachusetts	5.2		

II. Data and plan.

In this preliminary examination of the problem, undertaken to see whether a more detailed statistical analysis would be warranted, the following data were extracted on cards for 460 cities of a population of 10,000 or over in 1910, in the Registration Area for deaths. The figures were taken from the 1910 Census (Population) and Mortality Statistics for 1910. The "subscript numbers" are those which will be used in that position to designate the variables in the correlations.

Subscript number.	Variable.
1*a*	Death rate (crude) from all causes.
1*b*	Death rate (crude) from typhoid fever.
1*c*	Death rate (crude) from measles.
1*d*	Death rate (crude) from whooping cough.
1*e*	Death rate (crude) from diphtheria.
1*f*	Death rate (crude) from pneumonia.
1*g*	Death rate (crude) from diarrhea and enteritis (under 2).
1*h*	Death rate (crude) from puerperal fever.
1*i*	Death rate (crude) from tuberculosis of the lungs.
1*j*	Death rate (crude) from nephritis.
1*k*	Death rate (crude) from cancer.
1*m*	Death rate (crude) from suicide.
2.	Percentage of illiteracy.
3.	Population of city.
4.	Per cent. of negroes in the population.

The cards were used to form correlation tables relating the several variables to each other, and from the zero order correlation coefficients computed from these tables, there were calculated partial correlation coefficients of the form $r_{12\cdot34}$ by the usual methods (*cf.* Yule, 1907). All basic zero-order tables gave linear regressions.

It will be seen from the above outline of procedure that the results of this first study can in no wise be regarded as final. They merely indicate the general trend of the situation, and must be refined in several directions before they can be accepted as definitive. In particular it will be necessary: (*a*) to correct for differences in the age distributions of the populations; (*b*) to include data as to proportion of foreign born in each community; (*c*) to eliminate the element of spurious correlation (*cf.* Pearson, K., 1896, and Reed, L. J., 1921) which arises in the present study by virtue of the fact that in variables 1 and 2 the denominator of the rate fraction is identical, namely, the total population; and (*d*) to correct for geographical distribution of the cities included. We expect, in a much more detailed paper to follow the present one, to carry out all these refinements as well as certain others, but in the meantime it seems advisable, in view of the large amount of computing which must still be done before the final report can be finished, to put on record our preliminary rough findings. We feel confident from results already in hand that the picture presented in this paper will not be altered in the broad outline, by the more refined analysis, and at the same time we believe that the results are a distinct contribution to the general philosophy of public health administration in this country.

Regarding the official definition of illiteracy, the following may be quoted from the Census of 1910 (Vol. I, p. 1185):

> The population schedule for the census of 1910, like those for several previous censuses, contained two inquiries relating to illiteracy, namely, as to whether the person enumerated was able to read and as to whether he was able to write. Under the instructions to enumerators, ability to read or write in any language—not necessarily English—called for an affirmative answer. Answers to these questions were required only for persons ten years of age and over.
>
> For the present report, the Bureau of the Census has classified as illiterate all persons unable to write regardless of ability to read. A limited number of persons were reported as able to read though not able to write, but the statistics in regard to this class have not seemed of sufficient importance to call for a separate presentation. . . . In general, the "literate" population in this report should be understood as including all persons who have had even the slightest amount of schooling, while the illiterates represent persons who have had no schooling whatsoever.

III. Results.

1. *All causes.*

The pertinent net correlations are:

$$r_{1a_2 \cdot 34} = + .333 \pm .028$$
$$r_{1a_3 \cdot 24} = + .016 \pm .031$$
$$r_{1a_4 \cdot 23} = + .359 \pm .027$$

From these data it is seen that:

There is a moderately high, but entirely significant (coefficient $>$ 10 times its probable error) correlation between the death rate from all causes and percentage of illiteracy in these cities, when both absolute size of population and percentage of negroes in the population are held constant. In other words, just as we expected at the outstart, from *a priori* considerations, the degree of illiteracy of the population does appear to influence significantly its death-rate.

There is no significant correlation between the absolute size of a city and its death-rate, when the percentages of illiteracy and of negroes are held constant.

There is a significant positive correlation, as we should expect, between death-rate and percentage of negroes, when both illiteracy and absolute size of population are held constant. This is of the same order of magnitude as the net correlations between illiteracy and death-rate. It means that the negro is an important element in determining community death-rates, quite apart from his illiteracy.

2. *Typhoid fever.*

The coefficients are:

$$r_{1b_{2\cdot 34}} = -.046 \pm .031$$
$$r_{1b_{3\cdot 24}} = -.085 \pm .031$$
$$r_{1b_{4\cdot 23}} = +.180 \pm .030$$

There is no significant net correlation between the death-rate from typhoid fever and either percentage of illiteracy or absolute size of population. This is precisely what would be expected, because of the nature of the disease, and particularly the methods of its control. In American cities at the present time it can possibly mean but little, if anything, to an individual's chances of contracting typhoid whether he is intelligent or stupid as an individual. The control of the chances of contracting typhoid is essentially a community matter and is handled as such.

There is a small, but statistically significant net correlation between the typhoid death-rate and the percentage of negroes. At its face value this would indicate a greater racial susceptibility to this disease in the negroes than in the whites. Until a more complete analysis of the statistical data along the lines indicated above has been made, however, such a conclusion must be held in abeyance. as the existence of other factors not taken account of in the present study may bring about the result we have here.

3. *Measles.*

The coefficients are as follows:

$$r_{1c_{2\cdot 34}} = +.225 \pm .030$$
$$r_{1c_{3\cdot 24}} = -.097 \pm .031$$
$$r_{1c_{4\cdot 23}} = -.001 \pm .031$$

Between the death-rate for measles and percentage of illiteracy, there is a moderate positive correlation, which is, however, certainly significant statistically, being more than seven times its probable error. It is, therefore, probable that the extent of illiteracy in a given community plays a significant part in determining the death-rate from this cause.

On the other hand, the death-rate for measles does not appear to be significantly correlated with either the percentage of negroes in the population, or the absolute size of the city.

4. *Whooping cough.*

The net coefficients are as follows:

$$r_{1d_2 \cdot 34} = + .070 \pm .031$$
$$r_{1d_3 \cdot 24} = - .037 \pm .031$$
$$r_{1d_4 \cdot 23} = + .025 \pm .031$$

None of these coefficients can be regarded as significant in comparison with its probable error. Consequently it may be provisionally concluded that neither the degree of illiteracy in the community, nor the absolute size of its population, nor the percentage of negroes in the population, plays any significant part in determining a community's death-rate from this disease.

5. *Diphtheria.*

The correlation coefficients are:

$$r_{1e_2 \cdot 34} = + .345 \pm .028$$
$$r_{1e_3 \cdot 24} = + .064 \pm .031$$
$$r_{1e_4 \cdot 23} = - .204 \pm .030$$

The net coefficient between death-rate from diphtheria and percentage of illiteracy, with absolute size of population and percentage of negroes held constant, is the highest net coefficient of illiteracy that we have so far seen. It is more than ten times its probable error and is certainly significant statistically. This result, again, is in accord with what one would expect, since the mortality from this disease depends to such an extent on the manner in which the illness is treated medically.

There is no significant net correlation, when percentages of illiteracy and of negroes are held constant, between the death-rate from diphtheria and the absolute size of the city.

The apparently significant negative correlation between death-rate from diphtheria and percentage of negroes, when illiteracy and population are held constant, is the first significant negative coefficient that we have encountered. Taken at its face value, it would mean the probably absurd conclusion that the more negroes there were in a community of standard size and average intelligence, the smaller would be the death-rate from diphtheria. There is, however, no present justification for accepting this figure at its face value, because the magnitude and sign of the coefficient are almost certainly influenced considerably by certain variables, notably geographical distribution of the negro population, which are not taken account of in the present study. Therefore, no weight should be attached to this coefficient pending further detailed analysis.

6. *Pneumonia.*

The net coefficients here are as follows:

$$r_{1f2\cdot34} = + .463 \pm .025$$
$$r_{1f3\cdot24} = - .102 \pm .031$$
$$r_{1f4\cdot23} = + .031 \pm .031$$

Here the only significant net correlation is that between the death-rate from pneumonia and the percentage of illiteracy, when both size of population and percentage of negroes are held constant. This is the largest net coefficient we have yet encountered, and is nearly twenty times its probable error. It indicates that the average ignorance of the population is a distinct and highly significant factor in determining a community's death-rate from this disease. This is precisely what would be expected on the one hand, from the fact that the case fatality of pneumonia is influenced by the intelligence of the medical and nursing care of the case (*cf.* Pearl, R., 1919), and by the innate constitution of the patient, and, on the other hand, from the fact that any present practice of public health administration has so signally failed in the control of this disease.

The negative correlation between death-rate from pneumonia and absolute size of population, with percentages of illiteracy and of negroes constant, is suggestive, though it cannot be accepted in any way as final until more detailed studies are made. The coefficient itself cannot be regarded as certainly significant statistically, though it is probably so. It tends to indicate that the larger the city, other things being equal, the lower the death-rate from pneumonia. This result, however, may be largely due to the existence in the North of a few large cities, notably the boroughs of New York, with a relatively low pneumonia death-rate. This point can be settled when the geographical variables are included in the correlations.

The zero correlation between percentage of increase and the pneumonia death-rate, with other factors constant, appears to indicate that the higher absolute pneumonia death-rate in communities with large numbers of negroes is primarily to be attributed to the ignorance of the negro population rather than to any increased racial susceptibility. But, again, any positive conclusions on this point must await more adequate analysis of the facts.

7. *Diarrhea and enteritis (under 2).*

The coefficients are as follows:

$$r_{1g2\cdot34} = +.715 \pm .015$$
$$r_{1g3\cdot24} = +.046 \pm .031$$
$$r_{1g4\cdot23} = -.313 \pm .028$$

Here we have the highest net coefficient between the death-rate and illiteracy, the other factors of size of population and percentage of negroes being held constant. Again, this is precisely what would be expected. The death-rate from this cause depends so largely upon the intelligent home care of infants that one looks for exactly the result which emerges from this statistical study.

There is no correlation between absolute size of city and the death-rate in infants from diarrhea, the other factors being held constant, which is again an entirely reasonable result.

The fairly high and significant negative correlation between this cause of death and percentage of negroes of the population is in all probability a spurious result, which will disappear as other variables, particularly those of geographical distribution, are brought into the correlational nexus.

8. *Puerperal fever.*

The coefficients are as follows:

$$r_{1h2\cdot34} = +.048 \pm .031$$
$$r_{1h3\cdot24} = +.036 \pm .031$$
$$r_{1h4\cdot23} = +.029 \pm .031$$

The death-rate from this cause is not sensibly correlated with either illiteracy, absolute size of the city, or proportion of negroes in the population, the other factors being held constant in each case. With the exception of whooping cough this is the only one of the primarily environmental, infectious, communicable diseases which shows no significant correlation between illiteracy and mortality.

What the explanation is, is not as yet clear, and the whole question of the significance of this finding must be left until our more detailed study is completed.

9. *Tuberculosis of the lungs.*

We come now to the first of a group of diseases in which the constitutional factor unquestionably plays a more important part than in those which have so far been considered. The pertinent correlation coefficients are as follows:

$$r_{1i_{2\cdot34}} = +.010 \pm .031$$
$$r_{1i_{3\cdot24}} = +.094 \pm .031$$
$$r_{1i_{4\cdot23}} = +.400 \pm .026$$

There appears to be no significant correlation whatever between percentage of illiteracy and the death-rate from tuberculosis of the lungs, in these cities, when the percentage of negroes and absolute size of population are held constant. This result appears to emphasize the fact, for which there is now so much evidence from so many different sources, that fatal tuberculosis has, as a very large, if not indeed a dominant element in its causation, the inherited constitutional make-up of the individual. We believe that this coefficient will not be sensibly altered by the addition of more variables into the correlational nexus. This result should in no wise be interpreted as indicating that the intelligent cooperation of the patient is not an important factor in the prolongation of life of those stricken with pulmonary tuberculosis. Unquestionably, the intelligence of the patient does play a most significant rôle here, but there is no evidence that this element in the situation would sensibly affect the *death-rate* in a single sample taken at a given time, as is the case here.

There is, further, no evidence that the absolute size of city, other things being equal, has any sensible influence upon the death-rate from tuberculosis, and the zero coefficient which we find in this case is only what would be expected.

The high positive net coefficient between percentage of negroes in the population and the death-rate from tuberculosis, illiteracy and absolute size of city being constant, is precisely in accord with what is known as the greater susceptibility of the negro to tuberculosis.

10. *Nephritis.*

The coefficients are as follows:

$$r_{1j2\cdot34} = -.075 \pm .031$$
$$r_{1j3\cdot24} = +.124 \pm .031$$
$$r_{1j4\cdot23} = +.410 \pm .026$$

The net correlation between death-rate from nephritis and percentage of illiteracy is sensibly equal to zero, when the other factors are held constant. This is the expected result with a disease in which the constitutional make-up of the individual plays such a preponderant rôle in etiology as is the case with nephritis.

There seems to be a just barely significant correlation between the nephritis death-rate and the absolute size of city, when illiteracy and proportion of negroes in the population are held constant. Whether or not this coefficient can really be regarded as significant, and taken perhaps as an indication of the effect of the strenuous character of life in large cities, must be left for consideration when the results of a more penetrating analysis are in hand. Probably the correlation will disappear when other variables are taken into account.

The high coefficient between the nephritis death-rate and the percentage of negroes in the community probably indicates a really higher racial susceptibility to this cause of death, but, again, final judgment should be withheld until a more complete analysis has been made.

11. *Cancer.*

The net coefficients are:

$$r_{1k2\cdot34} = -.327 \pm .028$$
$$r_{1k3\cdot24} = +.029 \pm .031$$
$$r_{1k4\cdot23} = +.032 \pm .031$$

The significant negative net correlation between the cancer death-rate and the percentage of illiteracy is difficult to interpret. The *a priori* expectation would be that this coefficient should be small and positive, but still significant, on the ground that the most effective means of reducing mortality from cancer now known is extremely early operation, which in turn may reasonably be supposed to bespeak a rather high degree of intelligence on the part of the afflicted. It is possible that the negative coefficient arises through faulty diagnosis and registration. May it not be that deaths from cancer among the extremely ignorant elements of the population are usually not diagnosed or registered as such, but as something else? If this were to any considerable extent true, it would result in just such a negative correlation as we find here. It is possible that the addition of other variables, particularly those which involve the correction of age distribution of the population, may shift the result of this coefficient. Final judgment on the point must, therefore, in this case be reserved until the complete study is finished.

Neither absolute size of city, nor percentage of negroes in the population are significantly correlated with a death-rate from cancer, when the other two variables are held constant. This result seems entirely reasonable.

12. *Suicide.*

The coefficients are as follows:

$$r_{1m_{2\cdot34}} = -.179 \pm .030$$
$$r_{1m_{3\cdot24}} = +.100 \pm .031$$
$$r_{1m_{4\cdot23}} = +.076 \pm .031$$

Here the result, concerning the net correlation of illiteracy with the death-rate, is exactly what would be expected from Miner's (1922) detailed study of the suicide death-rate. The illiterate and the ignorant person generally is not so apt to commit suicide as is his more highly educated and intelligent brother. The net coefficient, while not large, is certainly significant. Neither of the other net coefficients can be considered as statistically significant.

IV. SUMMARY.

This purely preliminary study, by the method of partial correlation, indicates that the percentage of illiteracy in an urban community in this country is a significant factor in influencing unfavorably the death-rate of the community from all causes, and from certain particular causes, notably diarrhea and enteritis (under 2), pneumonia, diphtheria, and measles. The typhoid fever, whooping cough, and puerperal fever death-rates are apparently not significantly correlated with percentage of illiteracy, for probably quite different reasons in the different cases. Furthermore, there is apparently no significant net correlation between the death-rates from either tuberculosis of the lungs, or nephritis with percentage of illiteracy, presumably for the reason that in these diseases the innate constitution of the individual is an etiological fact of great importance.

All of the conclusions of this paper are to be regarded as in greater or less degree tentative until a much more intensive study of the problem has been completed, in which corrections will be made for a series of variables not taken into account in this preliminary survey of the field.

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OBSERVATIONS ON TWO STRAINS OF THE DIPHTHERIA BACILLUS.*

BY PERCY DAVOL MEADER.

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The value of diphtheria antitoxin as a therapeutic agent is too well established to require comment. There are, however, a certain relatively small number of cases which succumb to diphtheritic infection although they have received an ordinarily sufficient dose of antitoxin. Cultures obtained from cases of this character may be of considerable interest. Although it is generally believed that diphtheria antitoxin, as it is produced at present, will neutralize the toxin produced by any strain of *B. diphtheriae,* the claim has been made that this is not universally the case. Since there are cases in which antitoxin has been administered at the very outset of the infection and in large dosage, yet with fatal termination, any differences in the character of the toxin produced by the diphtheria bacillus might be expected to appear in cultures from these patients.

The observations of Havens (1920) showing two groups of the diphtheria bacillus also indicated some difference in the efficiency of antitoxin in neutralizing the toxins of the two types. More recently Paxson and Redowitz (1922), using strains from various sources, failed to note any significant difference in their toxic action when antitoxin was used for protection although they confirmed the existence of two immunological groups as reported by Havens. Two of the organisms included in the study of Paxson and Redowitz were obtained from this laboratory and have been under observation for nearly four years. They are of particular interest because of their history.

Through the courtesy of Dr. D. L. Richardson of the Providence City Hospital for Infectious Diseases, arrangements were made to obtain cultures from patients succumbing to diphtheria infection who had received antitoxin at the very outset of the disease in quantities usually ample to effect a cure. A series of eight or nine cultures were thus obtained from patients who had received the best possible treatment as regards hospital care and the early use of antitoxin, yet with fatal termination. After working over these cultures and testing their toxin formation under artificial conditions two strains, KL 4 and KL 6, were selected for thorough study since they appeared to produce a toxin not neutralized in the same ratio as other strains by standardized antitoxin. These are the cultures referred to in the paper of Paxson and Redowitz (1922).

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* From the Department of Bacteriology, School of Hygiene and Public Health, Johns Hopkins University.

As soon as these cultures were isolated in pure culture they were tested for virulence. Standard weight guinea pigs were injected subcutaneously with 1 c.c. of a saline suspension of the culture grown on Loeffler's blood serum. Each culture killed the animal in 18 hours, the autopsy showing typical lesions. During the four years these cultures have been under observation they have been tested for virulence at irregular intervals and have maintained their power of killing guinea pigs within 18 hours up to the present time.

Havens (1920) demonstrated by agglutination tests that there are two groups of *B. diphtheriae,* the first, of which Park 8 is a member, containing 82 per cent. of all the cultures studied. In order to classify the cultures used in this study rabbits were injected with Park 8, KL 4 and KL 6, respectively, and satisfactory agglutinating sera collected. Park 8 was used as the representative of group I because toxin prepared with this strain was used in the protection tests. Table 1 shows the agglutination reactions of the three strains to each of the sera.

The agglutination reactions were confirmed by absorption tests as follows: After the readings had been recorded the tubes were centrifuged until clear. To 0.5 c.c. of clear fluid taken from each tube in succession 0.5 c.c. of the homologous culture suspension was added. Homologous strains removed the specific agglutinins, and KL 6 removed agglutinins for both Park 8 and KL 4. It is evident from Table 1 that KL 4 belongs in a distinct group from Park 8 and may be placed in Havens' group II. KL 6 appears to be intermediate between groups I and II although Paxson and Redowitz placed it in the latter group. Twenty-two strains of *B. diphtheriae* isolated at various times from cases in Baltimore were likewise tested and of these 3, or 13 per cent., fell in group II. While no attempt has been made to group a large number of cultures the figures presented compare closely with those of Havens, who placed 18 per cent. of his strains in group II.

TABLE 1.

Agglutination tests with serum produced with culture Park 8.

	Strain.							Control.
	40	160	320	640	1280	2560	5120	
Park 8	+	+	+	+	+	+	−	−
KL. 4	−	−	−	−	−	−	−	−
KL. 6	+	+	+	+	−	−	−	−

Agglutination tests with serum produced with culture KL 6.

Park 8	+	+	+	−	−	−	−	−
KL. 4	+	+	+	+	+	−	−	−
KL. 6	+	+	+	+	+	+	−	−

Agglutination tests with serum produced with culture KL 4.

Park 8	+	−	−	−	−	−	−	−
KL. 4	+	+	+	+	+	+	+	−
KL. 6	+	+	+	+	+	+	−	−

During the past year a systematic study of the toxins of these two strains has been carried on. Toxin from the Park 8 strain has been used as the standard. When originally isolated KL 4 and KL 6 produced toxins of a strength of 0.005 and in one instance of 0.004. During the past year no toxin has approached this strength. We have no explanation to offer for this evident loss of toxin-producing ability. Berry and Blackburn (1912) report a study of toxin production by a large number of strains of *B. diphtheriae* in which there was a falling off in toxin-producing power after many generations on artificial media, but this was restored by a few transfers on broth containing ascitic fluid. For the purpose of this study no attempt to enhance toxicity was made and the strengths of the toxins shown in this study were very close to 0.01.

The two series of protection tests were conducted in the following manner. Standard antitoxin was obtained from the Hygienic

Laboratory through the courtesy of Dr. G. W. McCoy. One minimum lethal dose of each toxin was mixed in a tube with 0.001 of a unit of the antitoxin and allowed to stand for 15 minutes. The mixtures were then injected subcutaneously into standard weight guinea pigs. Duplicate tests of this character are shown in Table 2.

TABLE 2.

Protection tests with balanced toxin-antitoxin mixtures.

Strain.	Toxin dose.	Antitoxin.	Result in animal.
Park 8	0.01	0.01 unit	lived
Park 8	0.01	0.01 unit	lived
KL. 4	0.01	0.01 unit	lived
KL. 4	0.01	0.01 unit	lived
KL. 6	0.01	0.01 unit	lived
KL. 6	0.01	0.01 unit	lived

It appears that a balanced dosage of toxin and antitoxin has no harmful effect. It is possible, however, that the mixtures were so prepared that there was an excess of antitoxin, and a further set of experiments was undertaken wherein the antitoxin was held constant while the toxin was made an increasing variable. 0.25 of a unit of antitoxin was mixed in a tube with 25, 50, and 75 minimum lethal doses of each of the toxins and allowed to stand 15 minutes. Each animal was then injected subcutaneously. In every case the mixture was so arranged that each animal received 2 c.c. Table 3 shows the results of these tests.

TABLE 3.

Protection tests with antitoxin constant and increasing strength of toxin.

Strain.	Antitoxin.	Toxin.	Result.
Park 8	0.25 unit	25 m.l.d.	Animal lived
Park 8	"	50 m.l.d.	Animal lived
Park 8	"	75 m.l.d.	Animal died 48 hours
KL. 4	"	25 m.l.d.	Animal lived
KL. 4	"	50 m.l.d.	Animal lived
KL. 4	"	75 m.l.d.	Animal died 48 hours
KL. 6	"	25 m.l.d.	Animal lived
KL. 6	"	50 m.l.d.	Animal died 120 hours
KL. 6	"	75 m.l.d.	Animal died 48 hours

There appears to be no difference in the degree of protection afforded by the constant quantity of antitoxin to increased doses of toxin with the exception of KL 6. In this instance, although the animal receiving 50 minimum lethal doses died after 5 days, we would explain this as due to a greater suceptibility on the part of the guinea pig rather than to a difference in the type of toxin, since similar tests have not always reacted in this manner. The differences in the action of toxins KL 4 and KL 6, noted when the cultures were first isolated, were not found upon a later repetition of the work when standard antitoxin completely protected the guinea pigs. The possibility that these strains possessed greater invasive power than the group I strains was investigated. Guinea pigs were given 1, 1.5, and 2 units of antitoxin and after 60 minutes were injected with 0.25 blood serum slant of living bacilli suspended in salt solution. Table 4 shows the results of these tests.

TABLE 4.

Protective action of antitoxin against saline suspensions of cultures.

Strain.	Dose.	Antitoxin.	Reaction.
KL. 4......	1 c.c.	1 unit	Died 48 hours
KL. 4......	1 c.c.	1.5 units	Died 72 hours
KL. 4......	1 c.c.	2 units	Alive after 6 days
KL. 4......	1 c.c.	none	Died 18 hours
KL. 6......	1 c.c.	1 unit	Died 30 hours
KL. 6......	1 c.c.	1.5 units	Died 48 hours
KL. 6......	1 c.c.	2 units	Alive after 6 days; loss in weight
KL. 6......	1 c.c.	none	Died 18 hours

Two units of antitoxin appear to protect guinea pigs against the doses administered, although 1 and 1.5 units failed to prevent death. The rapidity with which unprotected animals have died suggests a high degree of virulence. There is no indication, however, of failure to protect on the part of the antitoxin, provided enough is used. The fatal termination of the infections of the patients from whom these cultures were obtained must be ascribed to some cause other than a difference in the type of toxin.

SUMMARY.

Two strains of *B. diphtheriae* are reported which were obtained from fatal cases, wherein antitoxin was administered early and in large quantities.

One strain falls distinctly into group II, as described by Havens; the other appears to be intermediate between groups I and II.

Protection against the toxin of these strains is afforded by standard antitoxin.

The strains are highly virulent and their action is neutralized by standard antitoxin.

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THE BIOCHEMICAL DIFFERENTIATION OF BACTERIA.*

By HOMER W. SMITH.

Charts I to XII.*

(Received for publication June 22, 1922.)

Introduction.

An enormous volume of literature has appeared in the last fifteen years on the use of various pure chemicals in the treatment of experimental and clinical infections. For the most part the experimental data are confined to more or less qualitative experiments on dye-stuffs, designed to determine whether or not a given substance possesses bactericidal powers. The number of substances so far studied is already running into the hundreds, and many investigators are at the present time engaged in elaborating and testing new ones.

After carefully considering the more important evidence available on the subject, it has appeared possible to make some deductions of a constructive nature; deductions which will not only serve to correlate the available data, but which may profitably guide further research for better chemotherapeutic agents. Moreover, since the normal and abnormal metabolic processes of the living cell are inseparably connected, the reactions of various types of cells to certain chemical agents should throw some light on the normal metabolic processes, which are undoubtedly very similar in bacteria and the higher forms of life. So the problem has significant aspects both in bacteriology and in general physiology.

Chemotherapy as a rational science had its beginnings in the work of Paul Ehrlich who was the first to study in a quantitative manner the relations between various therapeutic agents and diseased conditions in the animal body. Ehrlich's observations on the reactions between living cells and various chemical substances led him to postulate his well-known ''side-chain'' theory,—a theory of interest to us here principally because of its historical importance.

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* From the Lilly Research Laboratories, Indianapolis.

* The charts will be found at the end of the paper following the bibliographical references.

This theory was based on the concept of chemically reactive groups which Ehrlich called "chemoceptors." The vital staining powers of dyes, the reactivity of immune bodies, bactericidal substances and substances of a more or less complex nature which are in general poisonous for the living cell, were ascribed to these chemoceptors, it being supposed that there was a great degree of specificity in the nature of the chemoceptors possessed by the protoplasmic molecules in various cells and by the reactive substances (1). By postulating a sufficient number of specific chemoceptors it was a simple matter to explain any reaction or series of reactions which one might encounter in biology.

In so far as the general principles of this theory are concerned, there is no doubt that it has been an invaluable aid to the teaching of immunology, because it affords a simple means of elucidating complex immunological phenomena; it may even be said that in a few instances it has served to stimulate profitable research in this field. But in the light of more recent investigations, the notion of chemoceptors has fallen into disrepute. Even in immunology, where the theory is still frequently invoked as a pedagogical aid, it no longer exerts what could be called a directive influence on research. In chemotherapy there are, however, still many investigators who consider only the arrangement and nature of the chemical groups of a molecule when seeking an explanation of its bactericidal (or pharmacological) action. Those who were guided by Ehrlich's theory were not only led to confusion, but its enticing simplicity turned their attention from what are perhaps less obvious, but certainly much more important properties of the molecule than the structural formulae upon which it lays so much stress.

The chemoceptor theory presupposes that the activity of the substances with which it is concerned is primarily "chemical," or at least analogous to chemical reactions. As opposed to this theory, there are some who have gone to the other extreme and attempted to explain certain biochemical reactions on a purely physical basis. It is evident that no theory can adequately correlate the rapidly accumulating evidence in biochemistry of today, or serve as a fruitful guide to future research, which does not recognize the possibilities of both chemical and physical reactions in the normal and abnormal metabolism of the living cell. It is not intended, however, to support or advance any particular theory in this paper, because it appears at the present time that any attempt at an adequate treatment of the problem would constitute less theory than a mere recapitulation of facts.

Consequently the following pages include a review of the more important literature on bactericidal substances, supplemented by a few experiments designed to substantiate the deductions which are drawn during the course of the review.

I. THE TOXICITY OF STRONG, WEAK AND NON-ELECTROLYTES, OTHER THAN THE DYES.

1. *Factors relating to the environment.*

Robert Koch (2) was the first to instigate a systematic investigation of bactericidal substances. Following his discovery of the tubercle bacillus, he undertook the discovery of a disinfectant which could rid the body of this invader. Ehrlich and von Behring were closely associated with Koch at this time, and they were likewise led into this search for better bactericides. Ehrlich alone of the three persisted in the original idea—by virtue no doubt of his particular inclinations and training as an organic chemist—and his efforts met with success when he achieved the only substantial donation of synthetic chemistry to therapeutic medicine.*

At the time of the discovery of the bactericidal action of dye stuffs, the disinfectant powers of the commoner disinfectants, such as phenol, mercury bichloride, formaldehyde, alcohol, calcium hypochlorite and iodoform, had been extensively studied by Koch, v. Behring, Ehrlich and others of this school.

It was not long before investigators began to consider the physical-chemical aspects of the problems of disinfection. Thus it was pointed out by Dreser (1893) (3) that the low toxicity of the double hyposulphite of mercury and potassium could be explained by the fact that this salt on dissociation does not set free mercury ions, but breaks up into potassium and HgS_4O_6.

Kahlenberg and True (4) and Scheurlen and Spiro (5) confirmed Dreser's conclusions, the latter investigators extending the correlation between dissociation and disinfectant action to include certain compounds of iron. At the same time they showed that some mercury compounds (the ethyl chloride and ethyl sulphate) possessed strong disinfectant action apparently due, not to free ions, but to the undissociated molecule. They were also the first to show that the toxicity of mercuric chloride is decreased by the addition of sodium chloride, since it diminishes the concentration of free mercury ions.

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* The discovery of arsphenamine (or salvarsan) was, as every one knows, the result of a long series of "trial and error" syntheses performed under Ehrlich's direction. It is noteworthy that the "trial and error" method dominated the research of this period, and that little effort was ever made by Ehrlich or his coworkers to investigate the *modus operandi* of any substance of known efficacy.

The relation between dissociation and toxicity of strong electrolytes was put on a sound quantitative basis by the work of Krönig (7) and Paul (6) (1896). The details of their investigations were arranged with the greatest care, and they showed that in general the poisonous action of various acids, salts and bases ran parallel to the degree of dissociation. They found that salts of certain metals were less active when dissolved in alcohol or ether than when dissolved in water, a fact which they attributed to the decreased dissociation in the former solvents. This observation was confirmed by Minervini (8) on chromic acid, mercuric chloride and silver nitrate. Subsequently Mathews (9) concluded from evidence derived from the toxicity of various metal salts for Fundulus eggs that the action of these salts upon the protoplasmic system is a function of the ions of the salts. He extended the problem by his contention that the toxicity of any ion is a function of the solution tension of that ion. Consideration of this point will be reserved for another time, however.

The problem of the action of weak electrolytes proved not so simple.

Thus Krönig and Paul noted several exceptions to their dissociation law, particularly among the organic acids, where it was evident that the undissociated molecule possessed a distinct activity.

Overton (10) at this same time observed that salts of weak bases (alkaloids and dyes) with weak acids penetrated *Spirogyra* cells more readily than salts of strong acids, and that the addition of acids to salt solutions decreased or prevented penetration. Overton pointed out that the penetration and toxicity were roughly proportional to the amount of free base in the solution. Later Robertson (11) studied the solubility relations of a number of dyes and their penetration powers for mammalian tissue cells, and in a general way confirmed Overton's conclusions without agreeing with his interpretation. He found that basic dyes penetrate more readily in alkaline solution and acid dyes in acid solutions. Winslow and Lockridge

(12) found that the bactericidal power of hydrochloric and sulphuric acid ran parallel to the hydrogen ion concentration, while acetic and benzoic acid were destructive in concentrations at which they are only slightly dissociated. They noted that in the latter instance the effectiveness is specific for each acid, benzoic being five to ten times more toxic than acetic. There has subsequently been a great deal of work done on the influence of dissociation on the penetration of acid and basic substances into living cells. Unfortunately, there is not very good agreement between the results of various investigators and it is not practical to review all this work; for the details the reader is referred to Harvey (13), Crozier (14), Haas (15), Prowazek (16), Norton and Hsu (17), Traube (18), Euler (19), Tröndle (20), Collett (21), and Eisenberg (22). There is general agreement, however, that the effects of H-ion concentration on toxicity are due to the action on the drug, and not to any action on the cell; and that the toxicity of strong electrolytes (salts of the heavy metals, strong acids, etc.) is a function of their respective ions, while the toxicity of the weak electrolytes is a function of both the dissociated and non-dissociated fractions, the non-dissociated fraction playing, as a general thing, by far the predominant rôle.

The recent work by Crane (23) on this subject must be mentioned in some detail, for it constitutes the most accurate evidence available. Crane studied the influence of H-ion concentration on the toxicity of certain alkaloids for paramoecium. She concluded that the toxicity is a function of the undissociated base, and was able to show quantitative relationships between the toxic effects and the dissociation constants of the bases with which she worked. Thus the toxicity of caffeine and quinoline, bases so weak that only minute amounts exist as salts even in strongly acid solution, is little affected by the H-ion concentration, while bases of greater strength, such as strychnine, quinine and coniine, are more toxic by four to several hundred per cent. at P_H 8.0 than at P_H 6.0. Crane calculates that for quinoline, 99.2 per cent. of the total base is present as the free base at P_H 6.0, and 99.9 at P_H 8.0. This difference is insufficient to appreciably affect the toxicity. The relations for strychnine are quite different, the concentration of free base increasing from 11.5 per cent. to 53.7 per cent., and for piperidine, from 0.0008 per cent. to 0.086 per cent. with a change in P_H from 6.0 to 8.0. So it is evident that the influence of H-ion concentration on toxicity will vary with the nature of the substance under consideration, variations in the toxicity of the weak electrolytes being greater with strong acids and bases than with weak acids or bases.

If the influence of H-ion concentration is affected through changes in dissociation of the toxic agent and not through any effect upon the living cell, one would not expect the H-ion concentration to markedly affect the activity of non-electrolytes. Thus Davis (24) has found that phenol is equally active at P_H 6.5 and 8.0 against staphylococci and the colon bacillus. Graham-Smith (25) has observed that quinone is more active in acid than in alkaline media. He points out that if quinone is heated in the media, its bactericidal properties are destroyed. Thalheimer and Palmer (26) had already shown that quinone is many times more active than phenol, and much of the variation observed in the activity of various samples of phenol was attributed by them to the presence of quinone, since it is known to occur here as an impurity. Quinone is easily oxidized by atmospheric oxygen in the presence of organic material and this oxidation probably accounts for the loss of its activity on heating and may also account for the decreased activity with increasing alkalinity.

In contrast to the behavior of the true electrolytes, Beckman (27), Römer (28), Spiro and Bruns (29) have found that the toxicity of phenol is enhanced by the addition of sodium chloride. It is equally active in aqueous, alcoholic or ethereal solutions. Paul and Krönig (6) found that sodium phenolate is less toxic than phenol. Beckman has pointed out that in this case the undissociated molecule must be the active constituent. The more recent work of Miller (30), Lemon (31) and Laird (32) indicates that the effect of sodium chloride is attributable to a decrease in the ''chemical-potential'' or the potential solubility of the phenol in the presence of the salt, resulting in an increased tendency for the phenol to pass from the aqueous phase to a non-aqueous phase. Miller summarizes the relations which he observes as follows: ''Two solutions have the same toxicity, if they are such as to be in equilibrium with the same solution of phenol in an immiscible solvent.'' Undoubtedly Miller's conclusions form the basis of a generalization applying to the toxicity of non-electrolytes in general.

Factors relating to the cell.

In the next section it will be shown that weak electrolytes show a remarkable selective toxicity for Gram-positive bacteria as contrasted with Gram-negative bacteria. This selective action is not displayed by strong electrolytes or non-electrolytes. Thus Browning (33) states that the strong electrolytes, mercuric chloride, silver nitrate and gold chloride, are equally toxic for both types of bacteria. Certain non-electrolytes, phenol (Browning, 33), toluol (Benians, 34) and quinone (Graham-Smith, 25), are the only substances which have been stated to be more active against a Gram-negative than against a Gram-positive species.

In view of the extreme importance of this matter of the "selective" action of chemo-therapeutic agents, I have determined the sensitivity of a number of bacterial species to the bacteriostatic action of several typical strong electrolytes and non-electrolytes. The experimental procedure was as follows:

Agar containing 3 per cent. agar, 0.5 per cent. sodium chloride, 0.4 per cent. glucose, 1.5 per cent. peptone, and 0.5 pound veal extract to a liter, adjusted to P_H 7.3, was tubed in 10 × 130 mm. tubes, each tube containing 3 c.c. of agar. Dilutions of the reagents to be tested were made in 0.5 per cent. sodium chloride, one c.c. of these dilutions being added to 3 c.c. of melted agar, kept at 50° C. in a water bath. The seed cultures were raised on a 1 per cent. peptone solution, buffered with phosphates to P_H 7.3.* One tenth of a c.c. of a twenty-four-hour-old culture was always used for seed, the seed being added and mixed thoroughly with the agar-reagent mixture at 46–50°.

The tubes were then slanted and read after 24 and 48 hours incubation at 37° C. The saline and all flasks and pipettes used in the experiments were sterile.

The results of studies with copper chloride, cobalt nitrate, nickel nitrate, mercury bichloride, potassium arsenite and antimonyl potassium tartrate are given in Charts I to V inclusive. It is apparent that Gram-positive and Gram-negative species are about equally sensitive to the bacteriostatic action of copper chloride, cobalt nitrate, nickel nitrate and antimonyl potassium tartrate. There is more selective action in the case of mercury bichloride, and a very marked selective action in the case of potassium arsenite. In the latter instance, the Gram-negative group is on the whole the more susceptible.

As typical non-electrolytes, ethyl alcohol, acetone and phenol were chosen. The results of bacteriostatic experiments with these substance are given in Charts VI to VII inclusive. These experiments were repeated several times, and though the results of any one experiment were never exactly duplicated, their consistency clearly indicates that the organisms of the Gram-negative type are slightly more sensitive to the bacteriostatic action of non-electrolytes than are organisms of the Gram-positive type.

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* The composition of the phosphate-peptone media is given in Part IV.

II. The Toxicity of the Dyes.

The first observation of the bactericidal properties of dyes is usually accredited to Rozsahegyi (35) (1887), who called attention to the destructive action of carmine, methylene blue, gentian violet, vesuvin, fuchsin and methyl violet on several bacterial species. He demonstrated that certain organisms do not develop in the presence of certain dyes, and that there was a great degree of specificity or selection in the inhibitory action of various dyes on different bacteria. For two decades after Rozsahegyi's observations, discoveries in this field were mostly incidental, though several investigators noticed this selective action of dyes. Two of the earlier communications, by Penzoldt (36) and by Stilling (37) (1890), reiterate the fact that some dyes, particularly methyl violet, are very destructive to bacteria, but added nothing significant or new. Drigalski and Conradi (38) (1902) were apparently the first to suggest the practical utilization of this selective action in the isolation of bacteria from mixed cultures. They added crystal violet to their media to prevent the growth of cocci when recovering the typhoid bacillus from feces, as they found that this dye inhibited the growth of the cocci in question while not interfering with the growth of the typhoid bacillus. They also noted that the colon bacillus was more readily inhibited by malachite green and brilliant green than was the typhoid bacillus. It remained, however, for Loeffler (39) four years later to use the former dye as an aid to the separation of coli and typhoid strains. Shortly afterward Conradi (40) pointed out the superior advantages of brilliant green for this purpose. This selective action of brilliant green and other dyes has since become the basis of many important routine methods for the separation of coli, typhoid and other bacterial species, but since these methods have no particular bearing on our problem they will not be reviewed here. The bactericidal action of methyl violet was again mentioned by Dreyer, Kriegler and Walker (41) (1910), who drew attention to the fact that

Staphylococcus aureus was more susceptible than the typhoid bacillus, but this discovery was not followed by any extensive study of the dye.

During this period (1895–1912) a large number of dyes and other organic compounds were synthesized in Ehrlich's laboratory and tested, principally by *in vivo* experiments, on protozoal infections. If the bactericidal powers of these substances were ever extensively investigated, the data were never made public record, and but few contributions to our knowledge of bactericidal action have come from this source. A comprehensive review of the work done by Ehrlich and his coworkers is given in *Die Festschrift, Paul Ehrlich* (42).

It was twenty-five years after Rozsahegyi's observations before any systematic investigation was made of the selective action of dyes on bacteria. Churchman (43) observed that gentian violet inhibited the growth of *Bac. subtilis* at concentrations not inhibitive to *Bact. prodigiosum;* this led him to study the selective action of the dye on other strains. By the method of inoculation on divided plates, he extended his observations to include 334 strains representing 138 distinct bacterial species. The results showed that bacteria could be sharply divided into two groups according to whether or not their growth was inhibited by the dye. These groups he called respectively violet-positive and violet-negative. He noticed a close correlation between Gram-positive and violet-positive, and between Gram-negative and violet-negative organisms. He found about 90 per cent. correlation in the first case and 89 per cent. in the second. He thought that the selective action of gentian violet might be associated with its vital staining powers, because he observed that *Bac. subtilis,* which was very susceptible to the destructive action of the dye, was readily stained by it, while *Bact. prodigiosum* was both resistant to staining and to its destructive action.

In a second communication, in collaboration with Michael (44), Churchman points out that he was able to distinguish a particular strain of *Bact. enteritidis* from four others by its differing behavior toward gentian violet, while in all other respects the five strains were indistinguishable. The four strains of *Bact. enteritidis* were dye-resistant; the anomaly was dye-sensitive. In a third communication (45) Churchman stated that dahlia, parafuchsin, fuchsin, pararosanilin and rosanilin, substances chemically related to gentian violet, gave much the same results so far as inhibitive and selective action were concerned.*

Crossley (1920) (46) has recently shown that the commercial preparation of gentian violet is a mixture of dextrine and dye in about equal proportions; the dye is a mixture chiefly of hexa-methyl-p-rosanilin hydrochloride and pentamethyl-p-rosanilin hydrochloride with small quantities of lower homologs, particularly the tetramethyl compound.

He further established the fact that ten bacterial species were consistently positive or negative to the commercial mixture and its several dye components, in so far as inhibition was concerned. The organisms found to be gentian positive (or susceptible) were also Gram-positive.

The next systematic study of the bactericidal action of dyes was that of Simon and Wood (47) (1914), who studied the action of some sixty dyes on as many strains (comprising nineteen species) of bacteria. Their method was essentially the same as that of Churchman; that is, they worked with a fixed concentration of all dyes in agar, determining what species would grow after surface inoculation.

They attempted to relate bactericidal power to chemical composition and their conclusions are very important. They found that the inhibitory effect which the violet dyes, such as gentian violet, methyl violet, dahlia, etc., exercised on certain bacteria was not referable to their violet color, but to their chemical structure as triamino triphenyl methanes, and that it was intimately associated with the basic nature of the dye. This inhibitory power is not an exclusive property of triamino triphenyl methanes, however, but may be possessed to a greater or lesser extent by other strongly basic dyes. Acid dyes, irrespective of their chemical structure or color, did not possess inhibitory powers. Their work bears out Churchman's correlation between the susceptibility of Gram-positive bacteria and the relative insusceptibility of Gram-negative bacteria to the action of basic dyes.

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* Churchman was aware of Drigalski and Conradi's work and he points out that ''the important lead as to the selective action of crystal violet was not followed up by a systematic study of the dye.'' He apparently was unaware of Dreyer, Kriegler and Walker's observations published two years before, since he does not mention them. Though others have precedence over Churchman in the discovery of the selective action of dyes in general, and even of gentian violet in particular, nevertheless his observation was original and he was the first to appreciate the significance of this observation and to make a thorough investigation of the selective action of this and related dyes. His work remains essentially unamended to the present time.

Simon and Wood confirmed Churchman's observation that in a given species certain strains may behave anomalously in being more sensitive or more resistant to the action of a dye than other strains. They observed some instances of spontaneous reversal in behavior; a dye-resistant streptococcus strain became dye-sensitive, and a dye-sensitive strain of *Bact. enteritidis* became dye-resistant. They attributed these variations in behavior to the influence of variations in the culture media. They were able, in fact, to induce marked resistance in bacteria, originally sensitive to crystal violet, by exposure to sub-lethal concentrations of this substance. A similar observation was made simultaneously by Shiga (48) in Ehrlich's laboratory, where it had been observed that trypanosomes could acquire marked resistance by exposure to sub-lethal doses of dyes and other therapeutic agents. Following the theory held by Ehrlich and his coworkers, Simon and Wood attributed the inhibitory action of the dyes in question to the existence of ''receptors'' in the microörganism to which the dye was anchored, this combination supposedly leading to impairment of normal metabolism. Consequently they explained natural or acquired adaptation on the basis of the production of an excess of these receptors, or of receptors of varying kinds.

At this same time Browning and his coworkers (49) were studying the inhibitive effects of brilliant green. They observed marked differences in susceptibility to this dye between *Bact. paratyphosum, Bact. typhosum, Proteus vulgaris* and *Bact. coli,* the susceptibility increasing in the order named. This observation led them to recommend the use of brilliant green in liquid media for the separation of *Bact. typhosum* and *Bact. coli* (50), a procedure suggested by Löffler's use of malachite green for this same purpose.

Simultaneously with Simon and Wood's work, Krumwiede and Pratt (51) (1914) confirmed Churchman's division of bacteria on the basis of dye-susceptibility into two groups corresponding in general to their reaction to the Gram stain. These investigators emphasized the finding that the streptococcus and pneumococcus groups differed from other Gram-positive bacteria in that they showed greater resistance to gentian violet and allied dyes. They also point out that decolorization with sodium sulphite robs the dyes of some of their inhibitory powers. In a second communication they give the results obtained with several green dyes, the principal of which was brilliant green. From their data it is evident that, as Simon and Wood claimed, the bactericidal action of a dye is in no way referable to its particular color. Neither is its selective action, because the green dyes show the same selective action on Gram-positive bacteria as do the violet dyes. They also confirmed and elaborated Browning, Gilmour and Mackie's method for the isolation of the typhoid bacillus by brilliant green.

Browning and his coworkers were the first to call attention to the bactericidal action of acriflavine (52) (flavine, trypaflavine) and proflavine (53), the former compound having been synthesized in Ehrlich's laboratory in the study of trypanocides. These substances were said to be more active in the presence of serum than in saline, in contradistinction to most basic dyes, the activity of which is greatly reduced by tissue fluids. Like other basic dyes, these are more active against Gram-positive bacteria than against Gram-negative bacteria.

It has similarly been found that Gram-positive bacteria are more susceptible to quinine and hydrocuprein derivatives (Traube (54), Bieling (55)), to iodine (Breinl (56)) and to mesohematin and to magnesium and manganese derivatives of mesoporphyrin (Kämmerer (57)).

E. G. Davis and his coworkers (58–62) have studied a number of dyes, mercury derivatives and compounds related to phenolsulphonphthalein with the intent to find a suitable urinary antiseptic. Davis (24) has recently published a summary of his findings for over 200 dyes. The results of this work bear out the fact that it is only among the basic dyes that bactericidal substances occur and that all dyes and related substances are more active for the Gram-positive organisms than for the Gram-negative. From his extensive list Davis recommends acriflavine, proflavine and chlor-mercury fluorescein as the only substances with practical possibilities for this purpose. More recently Young, White and Swartz (63) have reported very favorably on dibrom-oxy-mercury fluorescein (mercurochrome 220).

In the few instances where the influence of H-ion concentration has been studied on the bactericidal action of the dyes, similar results have been obtained as with other weak electrolytes. Thus Graham-Smith (25) has found that the inhibitory power of homoflavine in meat extract bouillon was decreased by acid and increased by alkali. He found that similar variations could be obtained by varying the amount of nutrient material. The variations in bactericidal activity resulting from the addition of acid or alkali were most marked in media containing a minimum amount of meat extract. Since the author did not determine or control the H-ion concentration, his results are not as instructive as they might be. It is evident, however, that homoflavine is more active in alkaline than in acid media. Crystal violet, acriflavine, brilliant green and basic fuchsin have been shown by Beckwith (64), by Davis and Harrell (59) and by Browning, Gulbransen and Kenneway (65) to be more active in alkaline solution. Acid fuchsin (Beckwith (64)) and the acid chlor-mercury fluorescein (Davis, White and Rosen (60)) are more active in acid solution. Every one of forty-four basic dyes studied by Davis (24) proved to be more active in alkaline urine than in acid urine, and to be more active against staphyloccoci than against the colon bacillus.

In the absence of an exhaustive quantitative analysis of these phenomena, it may be remarked that it will probably prove impossible to account for the variations in the toxicity of many dyes arising from variations in the H-ion concentration on the basis of the degree of dissociation alone. It is probable that in the case of colloids, variations in H-ion concentration are responsible for profound variations in colloidality, which in turn modify the toxicity to a great extent. Thus Evans and Schuleman (66) have shown that the toxicity of a large number of acid dyes of the trypan blue group is attributable to their colloid nature, and that the colloidality and stability of these substances is influenced by heat, light, H-ion concentration, etc. Similarly Langer (67) has argued that the bactericidal action of the acridine dyes is a function of the degree of dispersion and this obviously will be affected by H-ion concentration.

The most striking fact disclosed by the data on the bactericidal powers of various dye stuffs is the remarkable selective action which all bactericidal dyes appear to have on Gram-positive bacteria. It has already been pointed out that Churchman established this fact for gentian violet by an exhaustive study of 138 species, and Simon and Wood simultaneously extended the work on about two dozen species to include some sixty dyes. Since this earlier work, it has become increasingly evident that there is a definite connection between dye-sensitivity and Gram-positivity. From a practical as well as a theoretical point of view, this fact is of great significance. But the problem labors under the disadvantage that so far as Churchman's and Simon and Wood's work goes, the correlation is not perfect; there appear to be Gram-negative species which are dye-sensitive and Gram-positive species which are dye-resistant. It has been deemed advisable therefore, to perform experiments of a more quantitative nature, giving special attention to those species which appear to be exceptions to the general rule, in order that a more systematic correlation between these two biochemical properties may be established. The outstanding exceptions to the correlation between Gram-positivity and dye-sensitivity in Churchman's earlier work were *Neisseria gonorrheae, Pfeifferella mallei, Erwinia oleraceae, Clostridium welchii* and *Clostridium sporogenes.* The first three species named are Gram-negative and appeared to be dye-sensitive. The last two are Gram-positive and appeared to be dye-resistant. Subsequently Churchman isolated a naturally occurring strain of *Bact. enteritidis* (Gram-negative) which throughout continued cultivation remained dye-sensitive.

In planning the experiments listed below, an attempt was made to include a species representative of each of the important genera, especially those mentioned above.

The bacteriostatic action of several dyes was determined on 32 species by the method described in the previous section. Readings were made after 24 to 48 hours; it was seldom that any difference was observed between the 24- and 48-hour readings, but in all cases the 48-hour reading is recorded. The results obtained with crystal violet and brilliant green are given in charts IX and X.

That the Gram-positive bacteria are the more susceptible to the inhibitive action of these dyes is clearly evident. Attention is called especially to *Neis. catarrhalis* which, though Gram-negative, is dye-sensitive. The genus *Neisseria* comprises all the Gram-negative cocci, the other important members of which are *Neis. gonorrheae* and *Neis. meningitidis.* Attempts to determine the dye-sensitivity of these species were not successful because of the difficulty encountered in raising them on the standard media. Failure to grow in such a case cannot of course be attributed to dye-sensitivity. It is believed, however, that the reaction of *Neis. catarrhalis* can be taken to represent the reactions of the genus as a whole. This uniformity of reaction of the various members of a genus is well established by the experiments listed below and in other portions of this paper.

Clos. sporogenes is Gram-positive and dye-sensitive. *Sp. Metch-*

nikovii, Vibrio tyrogena and *Pfeifferella mallei* are Gram-negative and dye-resistant. Thus correlation is obtained in most of those cases where Churchman failed to get it, the only exception to correlation as indicated by these experiments being the genus *Neisseria*. Particular attention will be given to this point in subsequent paragraphs.

There remains the fact that there may occur within a given species a strain which reacts towards the dye anomalously; reference has already been made to Churchman's dye-sensitive *Bact. enteritidis* strain. Moreover more recent work by the same investigator indicates that there may exist within a given strain two types of individuals, one dye-resistant and one dye-sensitive (68). Stowell, Hilliard and Schlesinger (69) have found that of eighty-seven streptococci strains, all but five were inhibited by gentian violet. Included in this five were several Gram-negative strains, but there was no general correlation between Gram-negativity and dye-sensitivity.

Simon and Wood obtained through a process of acclimatization dye-resistant, Gram-positive strains, though Churchman and others have been unable to repeat this work. It is well known that protozoa and bacteria can acquire resistance to arsenic and other deleterious agents, and there is no a priori reason for believing that bacteria cannot acquire resistance to dyes. Phenomena of chemical adaptation are universally encounterd among all organisms which reproduce asexually, but until the modus operandi of these adaptations is better understood, we can only assume that dye-sensitivity and Gram-positivity are *interrelated,* but independent variables in the biochemistry of the cell.

III. THE MECHANISM OF GRAM'S STAIN.

Summing up, the correlation between dye-sensitivity and Gram-positivity is not perfect in view of the dye-sensitive, Gram-negative *Neisseria* and occasional naturally occurring or artificial strains which behave anomalously; but the fact that these properties of the cell are associated to so great an extent shows clearly that there is some interdependence of the factors underlying them. It is necessary, before attempting a further analysis of these factors, to inquire into the intimate nature of Gram's stain. Gram first described his method of staining in 1884, and though numerous modifications of technique have been recommended from time to time, the essential procedure still remains the same. It consists of staining bacteria which have been smeared and dried on a microscope slide with an aqueous gentian violet solution. A small amount of anilin or anilin sulphate is added to the dye to increase its staining power: this however is unessential to the reaction proper. After the excess of dye has been removed by washing in water, the stained bacteria are subjected to an iodine solution (iodine in potassium iodide) which in some manner acts as a mordant. The excess of iodine is then removed by washing, and the smear treated with 95 per cent. alcohol. The alcohol dissolves the "mordanted" dye from certain species of bacteria (Gram-negative), but fails to decolorize other species (Gram-positive). By counterstaining with a second dye, the former are shown in marked contrast to those which retain the gentian violet.

The question is why does one type of cell retain the mordanted dye while the other type permits the alcohol to extract it.

The earlier theories about the mechanism of Gram's stain supposed that the essential process was a chemical one. Thus Unna (70), one of the first to investigate it, believed that the dye was bound by the iodine to certain substances in the bacterial body in such a way as to render it insoluble in alcohol. Dryer, Scott and Walker (71) suggested that the substance responsible for the retention of the stain is a lipoid, because Gram-positive bacteria which have been extracted with lipoid solvents lose their power to retain the dye, while some Gram-negative bacteria tend to retain the dye after being treated with emulsions of lecithin. Tamura (72) extracted a lipoid from Gram-positive bacteria with which the dye readily combined, and Jobling and Peterson (73) found that the lipoid bodies extracted from some Gram-positive bacteria differ from those extracted from Gram-negative bacteria in that the former contain a much larger proportion of the unsaturated fatty acids, as indicated by their affinity for iodine. They found a relation between the resistance to peptic digestion and the content of unsaturated lipoids, but they pointed out that the bacterial lipoids show more marked fluctuations than other cell constituents, the quantity depending largely on the composition of the media and to some extent on the age of the culture; and the chemical quality, unsaturation, being subject to changing oxidative conditions. Breinl (56) has found that intact Gram-negative organisms absorb more iodine than Gram-positive organisms, a contradiction to the results of Jobling and Peterson on the extracted lipoids. Cruickshank (74) has recently pointed out that lecithin and cephalin will form stable combinations with water-soluble dyes, rendering them lipoid-soluble. It is likely that any lipoidal extract from bacterial cells would contain substances of this nature, and in view of the absence of correlation between lipoid content and reaction to Gram stain, such extracts tell us nothing about the property responsible for the retention of the dye.

On the physical side of the question it was asserted by Brudny (75) that Gram-positive bacteria, being permeated both by the dye and by the iodine, contain a considerable mass of the compound stain which is therefore relatively more slowly removed from them by solvents than in the case of Gram-negative bacteria which he considers are not penetrated by the iodine. Eisenberg (76) has shown that Gram's reaction is not dependent on the chemical nature of the dye, for it can be demonstrated with the deeply colored dyes of the acid class, as well as with the basic dyes. He also showed that mordants are not necessary for the demonstration of the property.

The most instructive observations in this direction are those made by Benians (77). This investigator concluded that Gram-positive organisms are completely permeated by the dye and the iodine, while Gram-negative organisms are permeated with difficulty. He calls attention to the fact already mentioned by Churchman (43) that live Gram-positive organisms stain and lose their motility more quickly when suspended in the dye than some Gram-negative organisms. Thus *Bact. coli* in the unfixed state is apparently not permeated by the dye at all, while the gonococcus is quickly stained. Benians shows that the retention of the iodine-dye compound by Gram-positive bacteria is dependent upon the nature and integrity of the cell membrane, for as soon as this membrane is injured or broken up, the stain is dissolved out by the alcohol, an injury far short of complete disintegration of the cell sufficing to set the stain free. The amorphous cellular substance of Gram-positive and Gram-negative bacteria shows no marked difference as regards its affinity for the dye. Nor does the cell substance show any greater affinity for the iodine-dye compound than for the dye itself, the former being removed more readily, if anything, by the alcohol from the dyed amorphous cell substance. Benians concludes that Gram-positivity is due to a physical state of the cell membrane, a condition rendering it impermeable to the iodine-dye compound.

Burke (78), in a recent critique of Gram's stain, has performed an obvious and decisive experiment. He points out that if Benians is correct in his theory, one should be able to reverse the staining process by using an alcoholic solution of the iodine-dye complex. The iodine-dye complex should penetrate the Gram-negative cells, but not the Gram-positive cells. By adding alkali to the stain and using saffranin 0 as a counter-stain he obtained the anticipated result; staphylococci were not penetrated by the iodine-dye, whereas *Neis. catarrhalis* and *Bact. typhosum* were.

This interpretation of Gram's stain on a basis of permeability alone is also applicable to the acid-fast property of the tubercle bacillus towards carbol-fuchsin, according to Benians (77) and Sherman (79). While the amorphous cellular substance is more easily stained than the intact cell, it possesses no particular affinity for the dye, being readily decolorized in weak acid solutions.

Similarly Henrici (80) has found that yeast cells retain Gram's stain more firmly than do Gram-positive bacteria, yet the injured cell or the amorphous material is readily decolorized. Henrici emphasizes particularly a peculiar phenomenon observed in autolyzing yeast cells. Bacteria, he points out, either retain the stain or they are completely decolorized, whereas yeast cells undergo a process of partial decolorization, i.e., they show one or more particles in the decolorized cell which hold the dye longer than the rest of the cytoplasm. He reviews the work of several previous investigators and comes to the conclusion that the ability of these granules to resist decolorization is in direct proportion to their ability to resist autolysis. He considers them to be of an albuminous nature and believes that the data support a chemical theory for the fixation of the dye rather than a physical one as formulated by Benians. It seems, however, that any theory dealing with a physical state should be just as applicable to the membranes of granules which are morphologically differentiated from the cytoplasm as to the external cell membrane, and that these phenomena support rather than contradict Benians' theory.

The yeasts and bacteria are the only classes of cells in which this property of Gram-positivity is generally observed. Sections of a blade of grass, a lettuce leaf and an onion stalk do not contain, according to Henrici, any Gram-positive elements. Moulds stain irregularly, isolated granules in the mycelia retaining the stain while large areas do not stain at all. Protozoa, spirochetes and malarial parasites are Gram-negative. In animal tissues the nucleus retains the stain longer than the cytoplasm, but all the tissue elements may be ultimately decolorized.

Summing up, it appears that Gram-positivity is due to some property of the bacterial cell which permits the ingress of the water-soluble dye, but does not permit the egress of the alcohol-soluble iodine-dye compound. On the other hand, the Gram-negative cell is not readily penetrated by the water-soluble dye, but is easily penetrated by the alcohol-soluble iodine-dye complex. In both cases, permeability is conditioned by the physical state of the components of the intact cell membrane. Looking at it in this light it is not so difficult to understand how, in partially autolyzed Gram-positive cells, in old cultures or in cultures maintained under adverse artificial conditions the power of retaining the stain can be lost. What is lost is a particular condition or physical distribution of the constituents of the cell membrane.

In view of the observation that Gram-positive bacteria are to a slight extent more easily permeated by gentian violet (in Gram's stain) it might be argued that their greater sensitivity is a result of this increased permeability. But as Churchman observed in the case of *Bact. enteritidis* and *Bact. coli,* a strain may be negative and not easily permeated by the dye and still be dye-sensitive. It is a common experience that some Gram-positive species, such as *streptococcus,* may become Gram-negative in old cultures, and may remain Gram-negative when cultivated for long periods of time on artificial media. Such strains do not simultaneously acquire dye-resistance. Furthermore it is not likely that observations made on the permeability of a cell to gentian violet or iodine-gentian violet (as in Gram's stain) indicate the reaction of that same cell to substances of a widely different nature, such as the acid dyes, organo-metallic derivatives, etc. Yet the sensitive character of Gram-positive organisms is not restricted to gentian violet, or to basic dyes or acid dyes, but seems to involve all substances in the chemical category of weak electrolytes. Hence it is extremely improbable that this sensitivity is dependent directly on those factors which determine permeability to the reagents of Gram's stain.

These two properties of the cell are so to speak independent variables, since some species consistently possess one of them independently of the other, and since other species—perhaps all species—may acquire or lose either one or the other of them as the result of environmental influences of which we know very little. We can only suppose, in view of their usual association, that they are indirectly related through some biochemical factor as yet unrecognized.

On considering the biochemical reactions of bacteria which might throw some light on this problem, the most promising suggestions were found in the H-ion concentration at which various species showed their maximum growth, the sensitivity to cytolysis by sodium hydroxide and to digestion by trypsin and pepsin.

IV. The Optimum H-ion Concentration.

From the available data it was apparent that the optimum H-ion concentration for the Gram-positive species lay on the alkaline side, and for the Gram-negative species on the acid side of neutrality. Inasmuch as the evidence was insufficient to establish this point satisfactorily, experimental work was undertaken to determine the optimum H-ion concentration for a large number of species, particularly those not previously studied.

The most extensive information in the literature on this point is given by Dernby (82), who gives figures and graphs showing the optimum P_H for about thirty species. Dernby's figures in general indicate an optimum of very wide range for almost every species, but if one may judge from his graphs, he believes that a much narrower optimum can be defined. His graphs are apparently given with a view to expressing an optimum of rather narrow range, and it has been assumed that where he gives two figures representing the limits of the optimum range one may safely take the mean of these figures as a first approximation towards a single figure. In fact, this same reasoning applies to nearly all the data available in the literature. Most investigators are only interested in approximating the optimum range for cultural purposes, and consequently it is invariably necessary to reanalyze their data if more precise results are desired. Since these data are of interest to us principally for their tabular value, it will not be attempted to review the dozen or more contributions which have been quoted, but reference is made to the original articles in Table I where the data are given.

In regard to the determination of the optimum H-ion concentration it is not practical to give the results of the growth experiments in detail, but the methods which have been used in determining this optimum P_H will be briefly described.

The culture medium contained the following initial ingredients:

Difco peptone	20.00 grams
KH_2PO_4	13.62 grams
Water	q. s. 950.00 c.c.

The peptone and phosphate were dissolved, the mixture boiled for ten minutes, filtered and then the filtrate made up to exactly 1,000 c.c. To 40 c.c. portions of this acid mixture, when cooled, were added the required amounts of N/4 sodium hydroxide to obtain the desired P_H, and the portion made up to 80 c.c. These separate portions were then autoclaved, for thirty minutes at 15 pounds, in small flasks and tubed at a later date. Hard glass Wassermann

TABLE I.

Group II	Optimum P_H.	Reference number.	Group I.	Optimum P_H.	Reference number.
Diplococcus pneumoniae	7.8	82	*Spirocheta Obermeieri*	7.2	93
	7.8	84	*Spirillum Metchnikovi*	7.5	
	7.5		*Spirillum rubrum*	7.4	
Erwinia solanisapara	7.5		*Vibrio tyrogena*	7.4	
Erwinia caratovora	7.5		*Vibrio cholerae-asiaticae*	7.2	82
Neisseria gonorrheae	7.6	83	*Vibrio phosphorescens*	6.9	
	7.3	90	*Hemophilus influenzae*	7.3	88
	7.3	82		7.0	82
Neisseria meningitidis	7.4	89	*Hemophilus pertussis*	6.85	
	7.4	82	*Pfeifferella mallei*	6.85	
	7.0	94	*Bacterium typhosum*	6.9	82
Neisseria catarrhalis	7.4			6.9	85
Acetobacter. xylinum	7.4			6.6	
Zopfius Zenkeri	7.4			6.4	86
Zopfius Zopfii	7.5				
Azotobacter. chroococcum	7.5		*Bacterium paratyphosum A*	7.0	85
Saccharyomyces ellipsoidus	7.5			6.7	82
Saccharyomyces cerevisaea	7.2				
Actinomyces bovis	7.4		*Bacterium paratyphosum B*	7.0	85
Actinomyces madurae	7.3			6.7	82
Corynebacterium diphtheriae	7.4	82	*Bacterium coli*	6.8	
	7.2	95		6.5	82
Corynebacterium Hoffmanii	7.4			6.5	87
Corynebacterium xerosis	7.4		*Bacterium pneumoniae*	6.8	
Streptococcus viridans	7.4				

TABLE I.—*Continued.*

Group II.	Optimum P_H.	Reference number.	Group I.	Optimum P_H.	Reference number.
Streptococcus gracilis	7.5		*Bacterium fecalis-alcali-*		
Staphylococcus pyogenes albus	7.4	82	*genes*	6.65	
	7.4		*Bacterium enteritidis*	6.60	
Staphylococcus pyogenes au-			*Bacterium acrogenes*	6.50	92
reus	7.4	82		6.50	
	7.3		*Bacterium dysenteriae*	6.45	
Staphylococcus tetragenus	7.4		*Pseudomonas fluorescens*	6.85	
Clostridium tetani	7.4	82	*Pseudomonas pyocyanea*	6.8	82
Clostridium perfrigens	7.3	82		6.8	
Clostridium putrificum	7.3	82	*Pasteurella pestis*	6.8	82
Clostridium histolyticum	7.3	82	*Erythrobacillus prodigio-*		
Clostridium canadiense	7.3	82	*sus*	6.5	
Clostridium sporogenes	7.3			6.5	82
	7.3	82	*Proteus vulgaris*	6.5	
Fusiformis acne	7.3			6.5	92
Bacillus butyricus	7.3			6.5	82
Bacillus anthracoides	7.0	82	*Trypanosoma*	6.0	91
	7.3				
Bacillus lactis	7.3				
Bacillus mesentericus	7.2				
Bacillus subtilis	6.7	82			
	7.2				
Bacillus anthracis	7.2	82			
Sarcina lutea	7.2				
Rhodococcus cinnabareus	7.2	82			
Rhizobium radicicola	7.2	96			
Mycobacterium tuberculosis	7.0	82			

tubes (1 × 10 cm.) were used in the experiments and the medium was reautoclaved for thirty minutes after tubing. In every case the P_H of the medium before and after tubing was carefully determined electrometrically. As a rule very little change was observed as the result of autoclaving in tubes. Several lots of peptone were used in these experiments, but the results obtained in the several batches were fairly concordant. The amount of alkali used is given below:*

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* The P_H values given are estimated from smooth curves obtained from averaging several experiments and are only of value for approximating the amount of alkali required to obtain a desired P_H under the conditions of the experiment.

c.c. N/4 NaOH per 40 c.c. medium	P_H	c.c. N/4 NaOH per 40 c.c. medium	P_H	c.c. N/4 NaOH per 40 c.c. medium	P_H
2.4	6.05	8.50	6.75	13.50	7.45
3.1	6.15	9.50	6.85	14.00	7.55
3.8	6.25	10.30	6.95	14.40	7.65
4.45	6.35	11.00	7.05	14.80	7.75
5.10	6.45	11.70	7.15	15.20	7.85
5.95	6.55	12.40	7.25	15.55	7.95
6.85	6.65	13.00	7.35	16.00	8.05

Twenty-four-hour-old cultures were always used for seed. These were raised on phosphate-peptone solution with a P_H of 6.7 or 7.3, depending on the nature of the organism. With every species, several types of experiments were made: sometimes the P_H tubes were inoculated with 0.1 c.c., and sometimes with a loop of the seed culture. The method of inoculation will depend to a great extent on the nature of the organisms under consideration—whether it grows rapidly and whether it grows heavily. The P_H tubes were inspected after 6, 18, 24 hours, etc., and graded by a system of +, ++, +++. With most species from four to six separate experiments were performed on separate occasions to assure accuracy, and the figures given in Table I represent the net results of these experiments, averaged to the nearest 0.1 P_H.

At this point several considerations of a general nature should be mentioned. In the first place these figures cannot be assumed to have absolute significance. Dernby has pointed out that the optimum P_H differs considerably when determined by growth on agar or in bouillon. The optimum range is greater on agar, a fact which appears reasonable, since diffusion is slower and the individual organism is better enabled to modify and combat its environment. It may also be reasoned that the optimum range will be narrower and perhaps different, under adverse cultural or nutritional conditions. But, on the whole, the results obtained by various investigators agree fairly well and indicate that such factors as these lead to insignificant differences. Schönholz and Meyer (85) have found that various strains of *Bact. paratyphosum* differ slightly in their optima, one strain consistently showing a sharp peak at P_H 6.5 and another a plateau curve from P_H 6.6 to 7.4. This leads them to suggest that bacteria may undergo a process of adaptation to the influence of acid and alkali. Graham-Smith (81) has found that by continuous growth in acid, neutral and alkaline meat extract the capacity of staphylococci to multiply, when transplanted into media of different reactions, is greatly altered. His results clearly indicate an acclimatization to reaction and a marked tendency to retain this acclimatization after transfer to new conditions.

The work of Cohen and Clark (92), Avery and Cullen (84), Jones (97) and others shows that the limiting H-ion concentration is not characteristic of a species, but varies considerably with different strains and with the nature of the culture medium.

I have studied only one strain in each case, and it must not be supposed that these results can be accurately reproduced on other strains. It must also be remembered that most species of bacteria grow profusely over a wide range and that it is difficult to determine a sharp optimum, even by the closest observation. But in view of the good agreement obtained by various investigators, the optimum H-ion concentration appears to be a characteristic and fairly constant species-index. I believe that the statement is warranted that a given species will grow better in acid or alkaline media, and in many cases an optimum may be indicated to within ± 0.1 P_H.

The results of this study, as listed in Table I, show again a division of bacteria into two great groups, correlating approximately with Gram's stain and dye-sensitivity. The Gram-positive bacteria, and the *Neisseria*, have optimum P_H values above 7.0—i.e., in alkaline solution. In this group the correlation between optimum P_H and dye-sensitivity is almost complete. The Gram-negative bacteria have optimum P_H values below 7.0—i.e., in acid solution, with the exception of the vibrios and the spirilla. The fact that these organisms are dye-resistant and Gram-negative, but appear to grow best in an alkaline medium, would indicate that they occupy an intermediate position between the two groups.

V. Sensitivity to Cytolysis by Sodium Hydroxide.

Kruse (98) calls attention to the fact that Gram-negative bacteria are more susceptible to the cytolytic action of sodium hydroxide solutions than are Gram-positive bacteria. He does not, however, give data on a large number of species, so I have investigated this point on the species available to me. The experimental procedure is given below, and the results in chart XI.

Suspensions were made by removing the growth from twenty-four-hour agar slants with 0.5 per cent. sodium chloride solution.

The dilutions of sodium hydroxide were also made with saline. One drop of the bacterial suspension was added to 1 c.c. portions of the various sodium hydroxide solutions and to 1 c.c. of saline as a control. The results were observed after two hours, though they were essentially the same after twenty-four hours. There was invariably a slight amount of lysis in every tube, due presumably to the presence of dead or partially autolyzed cells; this has been neglected in the table, because it is desired to emphasize the marked difference in the behavior of the two classes of bacteria. (This difference is nicely demonstrated by the use of a single dilution, i.e., 0.5 per cent. sodium hydroxide.)

With the exception of *Neis. meningitidis* there is excellent correlation between the optimum H-ion concentration and the sodium hydroxide lysis test. *Neis. catarrhalis* and *Neis. gonorrheae* are quite resistant to lsyis when compared with the members of the Gram-negative group. On the other hand it will be noted that the vibrios and spirilla are just as susceptible to sodium hydroxide lysis as the other members of the Gram-negative group.

VI. SENSITIVITY TO PEPTIC DIGESTION.

Kantorowicz (99) was the first to study the digestion of bacteria by pepsin and trypsin. He confirmed the older observation of the absence of digestion of the intact cell whether living or dead, and showed that heating Gram-negative organisms to 70° C. destroyed their power of resistance, while Gram-positive organisms would resist digestion even after boiling. Jobling and Peterson (73) have confirmed this observation by following the non-protein nitrogen in bacterial suspensions exposed to trypsin. They found that dried organisms resist digestion in a degree proportional to their content of unsaturated lipoids, but with live organisms they found, as in the case of Gram's stain, no exact correlation between the lipoid content and the rate of digestion. They concluded, nevertheless, that the intact bacteria resist digestion because of the absence of an exposed protein substrate, arguing that the proteins were protected by the lipoids.

Bacterial suspensions were made by removing the 24-hour growth from nutrient agar and suspending in saline. The suspensions were heated for one hour at 65° C.

A 0.5 per cent. solution of commercial pepsin was made in 0.3

per cent. hydrochloric acid. One drop of a heavy bacterial suspension was added to 2 c.c. of the pepsin solution contained in Wassermann tubes and incubated for 24 hours. Controls for comparison were made by adding one drop of the suspensions to 2 c.c. of 0.3 per cent. hydrochloric acid. Comparisons were made on a basis of opacity only.

The results of the peptic digestion test are given in chart XII. The differentiation of the two groups is not nearly so clean cut with peptic digestion as with sodium hydroxide lysis, but the results, nevertheless, show that the Gram-negative group of organisms is uniformly more susceptible to peptic digestion than is the Gram-positive group. In the latter, *Neis. meningitidis* showed no greater cytolysis than did *B. subtilis* or *Staph. tetragenus,* and *Neis. catarrhalis* and *Neis. gonorrheae* showed essentially no cytolysis whatever. In the Gram-negative group, the vibrios and spirilla were only partially digested, thus bearing out the supposition that in certain respects they occupy an intermediate position between the two groups.

SUMMARY.

It has been found that bacteria may be divided into two great groups, corresponding roughly to their reaction to Gram's stain. The comparative reactions of these groups are summarized in the following paradigm:

	Group I.	Group II.
1. Gram's reaction	Negative	Positive
2. Optimum P_H	Acid	Alkaline
3. Strong electrolytes	Equally sensitive	
4. Weak electrolytes (dyes)	Resistant [*]	Sensitive
5. Non-electrolytes	Sensitive	Resistant
6. Sodium hydroxide lysis	Sensitive	Resistant
7. Tryptic digestion	Sensitive	Resistant
8. Spores	Never (?)	Possible

[*] Churchman in a recent article (101) states that certain Gram-negative bacteria are more susceptible to the action of acid fuchsin than are the spore-bearing, Gram-positive bacteria. Churchman states that "while long exposure to the dye (acid fuchsin) at room temperature produces the reaction, a slight increase in temperature (to 45° C.) makes it much sharper and speedier." Since no data are given in this preliminary note, it is impossible to make further comment on this apparent contradiction. I may state, however, that I have tested many species on *acid fuchsin, erythrosin, eosin* and other acid dyes, and the Gram-negative group of organisms has invariably shown much greater resistance than the Gram-positive group.

1. Group I contains no Gram-positive bacteria. Group II contains the consistently Gram-negative *Neisseria.*

2. Group I contains the Family *Spirillaceae,* which show maximum growth at P_H 7.5–7.2. Group II contains no organisms showing maximum growth of P_H values less than 7.0.

3, 4 and 5. The sensitivity of various organisms to the bactericidal or bacteriostatic action of toxic agents is subject to wide variations within a species or even within a strain, due to factors unrecognized at the present time. The reactions indicated above are fairly consistent and quite typical, however. The toxicity of strong electrolytes appears to be a function of the dissociated fraction; the toxicity of weak electrolytes appears to be a function of the undissociated fraction.

6 and 7. The division into two groups on the basis of sensitivity to cytolysis by sodium hydroxide or to digestion by trypsin is not so clean cut as in the case of Gram's reaction, optimum P_H or sensitivity to the inhibitive action of the dyes. Though the exceptions are few they show no agreement with the exceptions observed in the other tests.

8. All bacteria which are accepted spore-formers are included in group II. Not all bacteria in group II form spores, however.

The above properties appear to be independent variables, in the sense that any one of them may be possessed by a particular strain of bacteria, independent of the other properties of that group.

Nevertheless, the consistency with which these properties are associated in the vast majority of cases indicates that they are indirectly related through some as yet undefined biochemical factor in the cell.

A close analysis of the mechanism of Gram's stain; coupled with what we know of cytolysis in general, makes it appear that this determining factor resides in the cell membrane and that it is due to quantitative differences in the normal physical state of the constituents of this membrane; qualitative differences in the constituents of the membranes of the two types of cells are not excluded however.

Further discussion of the possible explanation of these phenomena will be deferred until other evidence bearing on the equilibria in protoplasmic membranes has been considered.

TABLE II.

Group I.	
Bacterium capsulatum	0
Bacterium pullorum	0
Bacterium murium	0
Bacterium alcaligenes	0
Bacterium icteroides	0
Bacterium coscorova	0
Bacterium cholerae suis	0
Bacterium astheniae	0
Bacterium melitensis	+
Bacterium cloacae	+
(2 strains)	0
Bacterium Sternbergii	+
Erythrobacillus indicus	0
Erythrobacillus fuchsinus	0
Erythrobacillus rutilescens	0
Erythrobacillus rosaceus metalloides	0
Erythrobacillus Kiliensis	+
Pseudomonas violacea	0
Pseudomonas Frostii	0
Pseudomonas cyanogenes	0
Pseudomonas aquatilis	+
Vibrio cholerae-asiaticae	0
Vibrio proteus	0
Vibrio danubicus	0
Vibrio phosphorescens	0
Vibrio Milleri	0
Proteus mirabilis	0
(2 strains)	+

Group II.	
Bacillus pseudo-anthracis	+
Bacillus megatherium	+
Bacillus mycoides	+
Bacillus liodermos	+
Bacillus ramosus	+
Bacillus tetani	+
Bacillus vulgatus	0
Micrococcus citreus	+
Micrococcus citreus agilis	+
Micrococcus versatilis	+
Micrococcus luteus	+
Micrococcus flavus	+
Rhodococcus agilis	+
Sarcina rosea	+
Sarcina flava	+
Sarcina aurantiaca	+
Sarcina ventriculi	+
Sarcina mobilis	+
Erwinia amylovora	+
Erwinia oleracea	+
Erwinia melonis	0
Rhizobium radicicola	+
Leptotrichia buccalis	+
Erysipeolthrix rhusiopathiae	+
Clostridium amylobacter	+
Clostridium Welchii	0
Clostridium anthracis symptomatici	+
Clostridium edematis maligni	+
Lactobacillus bulgaricus	+
(2 strains)	0
Streptococcus endocarditidis	+
Mycobacterium tuberculosis	
(2 strains)	+
(2 strains)	0
Mycobacterium Moelleri	0
Mycobacterium smegmatis	0

The Classification of Bacteria.

It is significant that this division of bacteria into two groups, on the basis of biochemical reactions, accords nicely with the classification into Families, Genera, etc., on the basis of morphological, cultural and immunological characters (102). Reference to Table I shows that all the *Bacteriaceae* studied are in group II, while all the *Bacillaceae* are in group I. As a tentative measure, Churchman's data on sensitivity to gentian violet may be utilized to extend the list given in this communication to further species. Those species which were studied by Churchman and which are not included in the present investigation are listed in Table II. Group I is the dye-resistant group, and group II is the dye-sensitive group (+ = dye-sensitive; 0 = dye-resistant).

Combining Churchman's data with those given in Table I, it will be seen that there is in general a very marked tendency for the various species of a given genus to behave alike. The failure of an organism to grow in the presence of the dye is a much less certain index of its nature than growth where inhibition would be expected. It must be kept in mind that when Churchman performed these experiments he had in mind only the correlation between Gram-positivity and dye-sensitivity. I have shown that in several instances (*Vibrio tyrogena, Vibrio phosphorescens, Spirillum Metchnikovii, Spirillum rubrum, Pfeifferella mallei,* etc.) reinvestigation clears up the contradiction.

With the exception of the few anomalies that remain, this division into two great groups not only separates the genera intact, but also the families. This is brought out in the paradigm below:

Group I.	*Group II.*
Gram-negative	Gram-positive (*)
Optimum $P_H<7.0$ (†)	Optimum $P_H>7.0$
Sensitive to cytolysis by alkali	Resistant to cytolysis by alkali
Sensitive to peptic digestion	Resistant to peptic digestion
Dye-resistant	Dye-sensitive
	D. ACTINOMYCETALES (‡)
	I. Actinomycetaceae
	II. Mycobacteriaceae

* Except *Neisseria,* which are Gram-negative.

† Except *Spirillaceae,* which have an optimum P_H above 7.0.

‡ It is probable the Group II contains besides the yeasts, the higher plant cells, since they are resistant to NaOH cytolysis and peptic digestion. Animal cells, on the other hand, such as spirochaetes, trypanosomes and the higher protozoa, are probably related more closely to Group I. It is interesting to note that in vitro cultures of embryo tissues are relatively dye-resistant, as are also the protozoa (100).

	E. EUBACTERIALES
	I. Nitrobacteriaceae
II. Pseudomonadaceae	←———————
III. Spirillaceae	←———————
	IV. Coccaceae
V. Bacteriaceae	←———————
	Erwinia
3. ———————→	Zopfius
4. ———————→	Lactobacillus
———————→	VI.
	Bacillaceae

The order ACTINOMYCETALES apparently contains no organisms belonging to group I. The order EUBACTERIALES is divided,—families II, III and V being in group I, and families I, IV and VI in group II. The only family to be split is the *Bacteriaceae;* all the genera in this family belong to group I, with the exception of *Erwinia, Zopfius* and *Lactobacillus,* which belong to group II.

This almost clean separation of the families of bacteria on the basis of biochemical reactions cannot be without significance. It suggests that closer study of the immunological and morphological characters in connection with the biochemical characters would prove both interesting and valuable.

A more detailed discussion of the possible chemical factors involved in these two opposed types of bacterial membranes will be postponed until it has been possible to present other evidence bearing on protoplasmic membranes in general.

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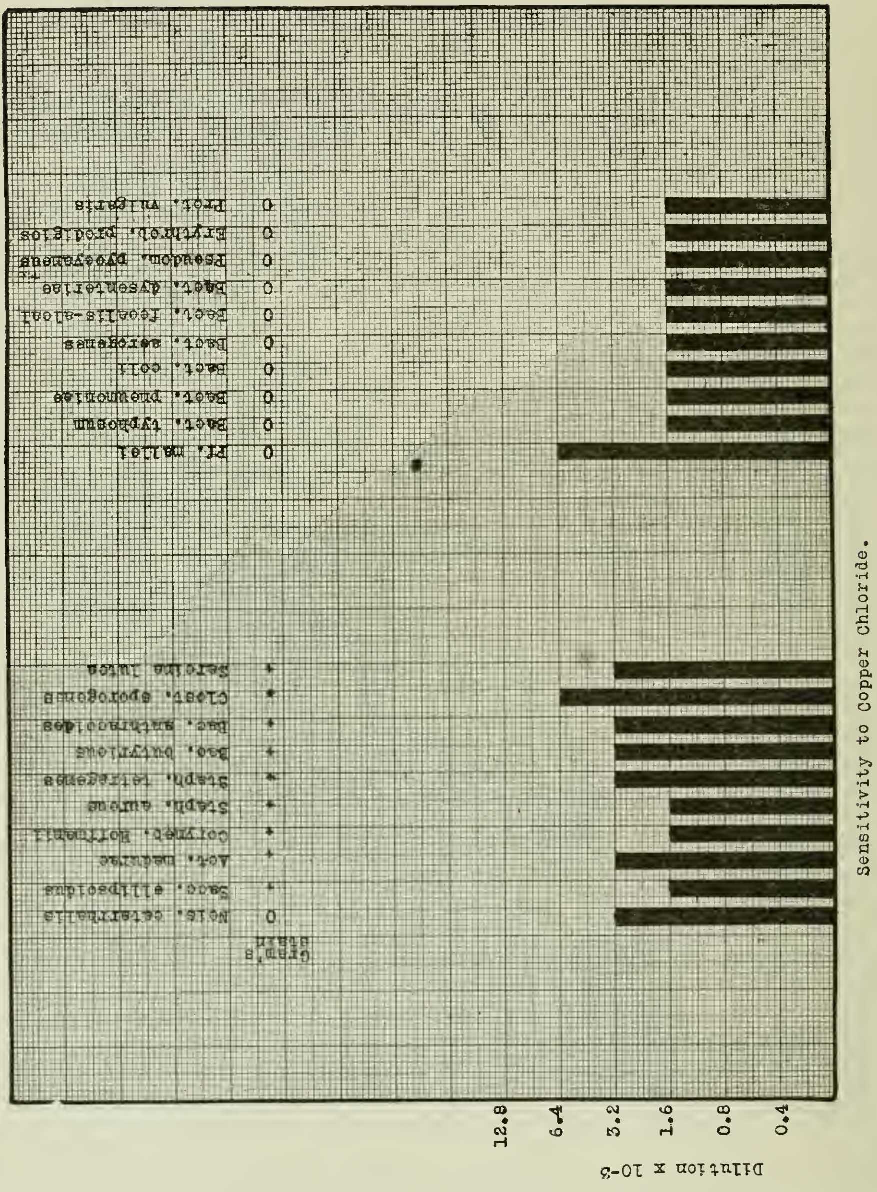
CHART I.



Sensitivity to Copper Chloride.

CHART II.

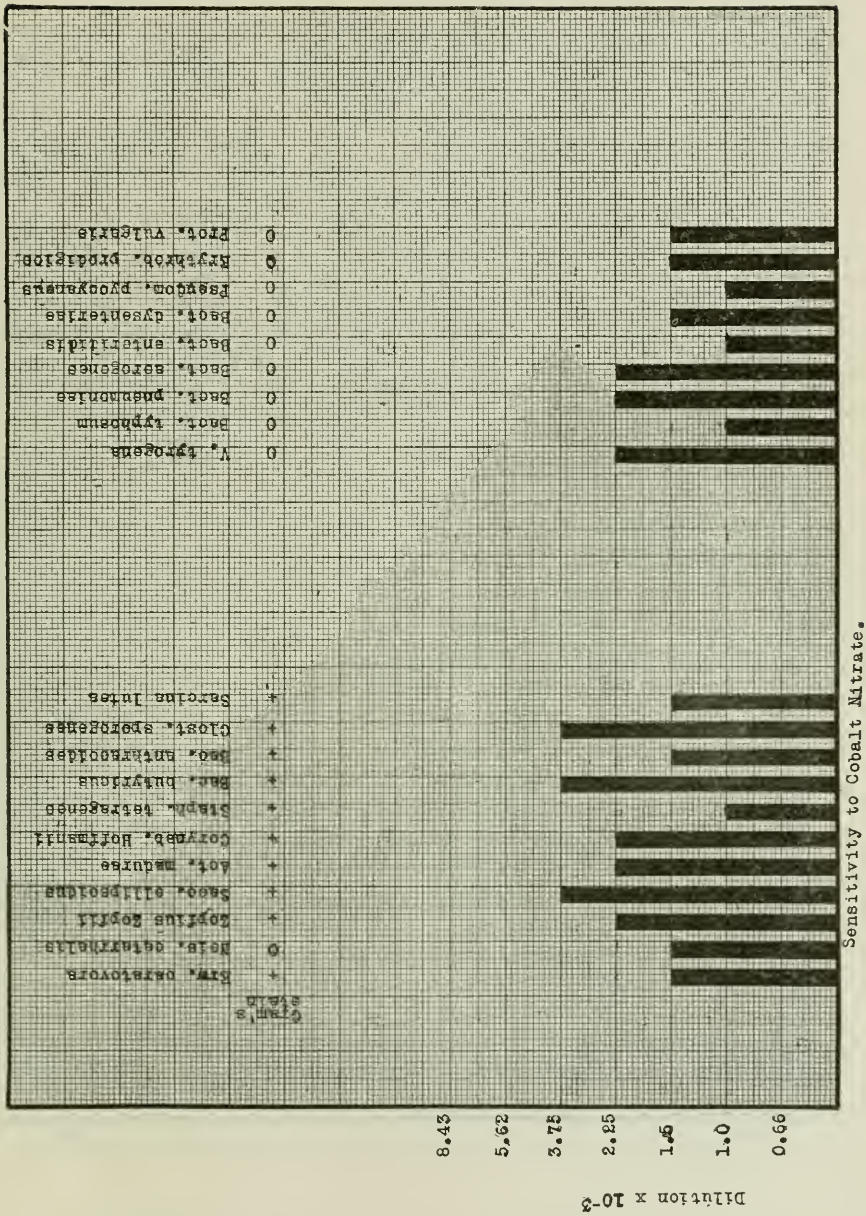

Sensitivity to Cobalt Nitrate.
Gram's stain
Erw. carotovora +
Mon. cetartheleia 0
Zopfius Zopfii +
Sacc. ellipsoidus +
Act. madurae +
Coryneb. Hoffmanii +
Staph. tetragenes +
Bac. butyricus +
Bac. anthracoides +
Clost. sporogenes +
Sarcina lutea +
V. tyrogenus 0
Bact. typhosum 0
Bact. pneumoniae 0
Bact. aerogenes 0
Bact. enteritidis 0
Bact. dysenteriae 0
Pseudom. pyocyaneus 0
Erythrob. prodigios 0
Prot. vulgaris 0
8.43
5.62
3.75
2.25
1.5
1.0
0.66
Dilution x 10-3

CHART III.

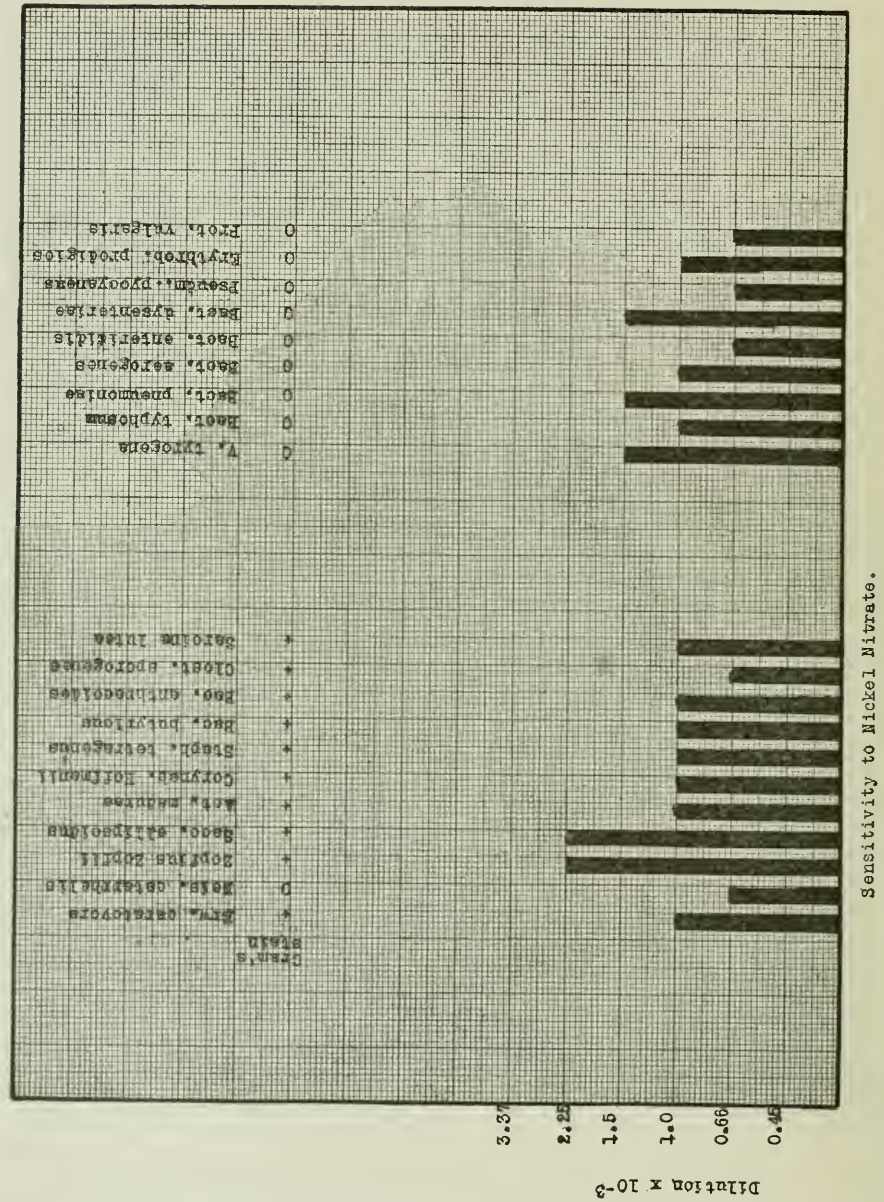

Sensitivity to Nickel Nitrate.
Dilution x 10^{-3}
0.45
0.66
1.0
1.5
2.25
3.37
Gram's stain
+ Erw. carotovora
0 Neis. catarrhalis
+ Zopfius Zopfii
+ Sacc. ellipsoideus
+ Act. madurae
+ Corynb. Hoffmanii
+ Staph. tetragenus
+ Bac. butyricus
+ Bac. anthracoides
+ Clost. sporogenes
+ Sarcina lutea
0 V. tyrogena
0 Bact. typhosum
0 Bact. pneumoniae
0 Bact. aerogenes
0 Bact. enteritidis
0 Bact. dysenteriae
0 Pseudm. pyocyaneus
0 Erythrob. prodigios
0 Prot. vulgaris

CHART IV.

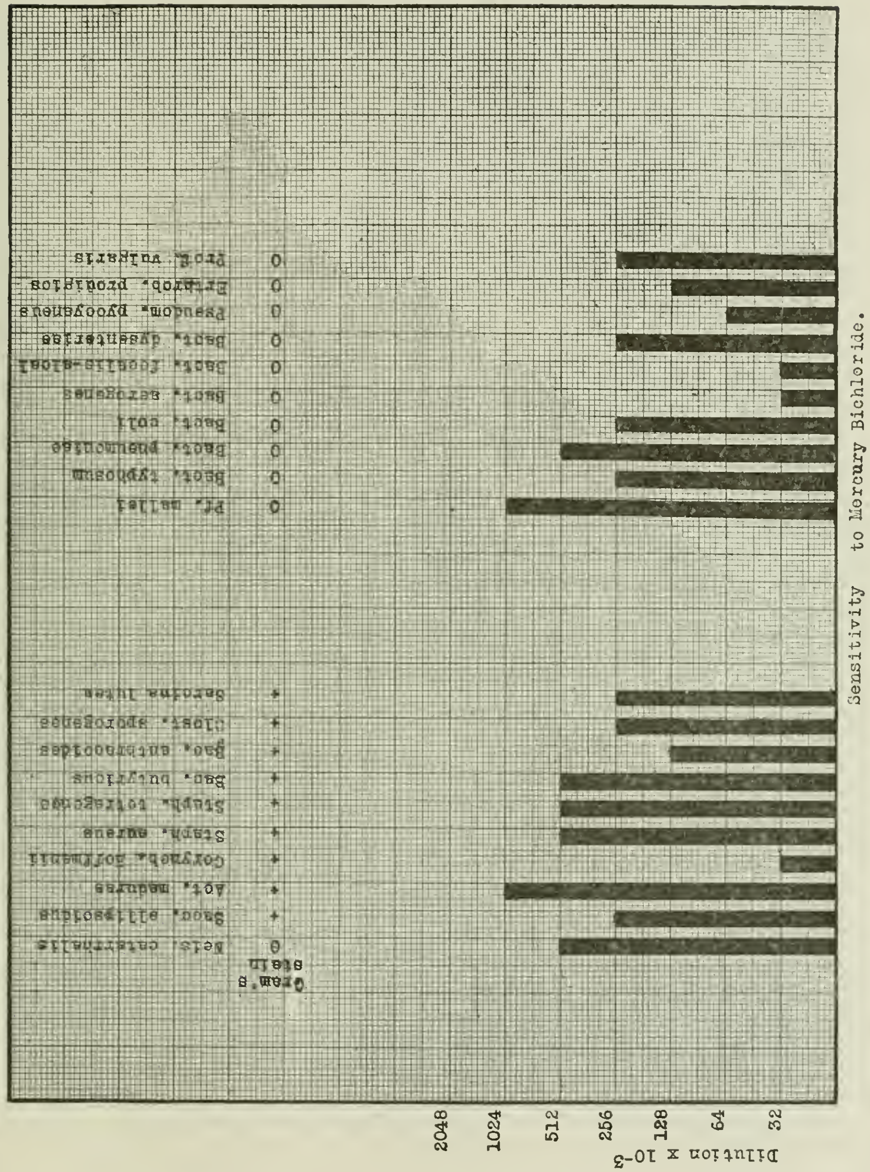
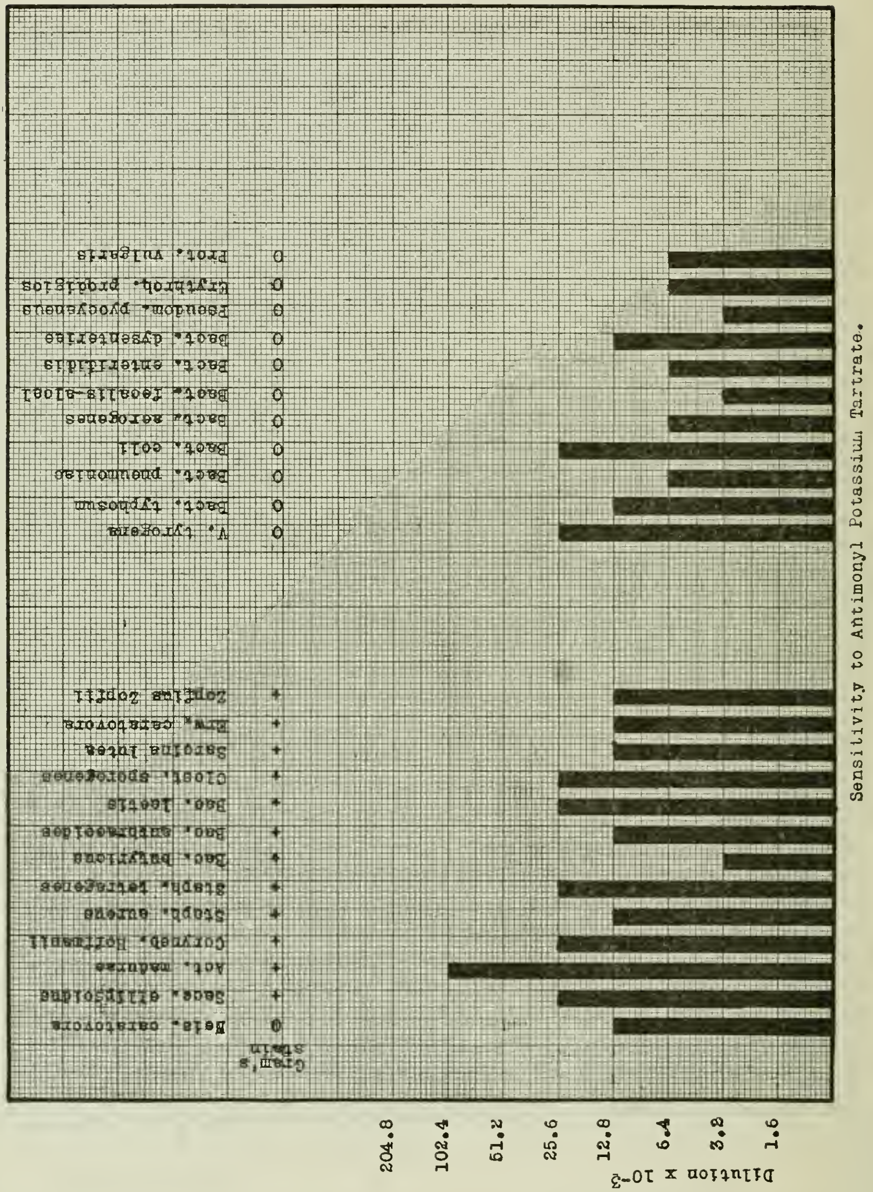
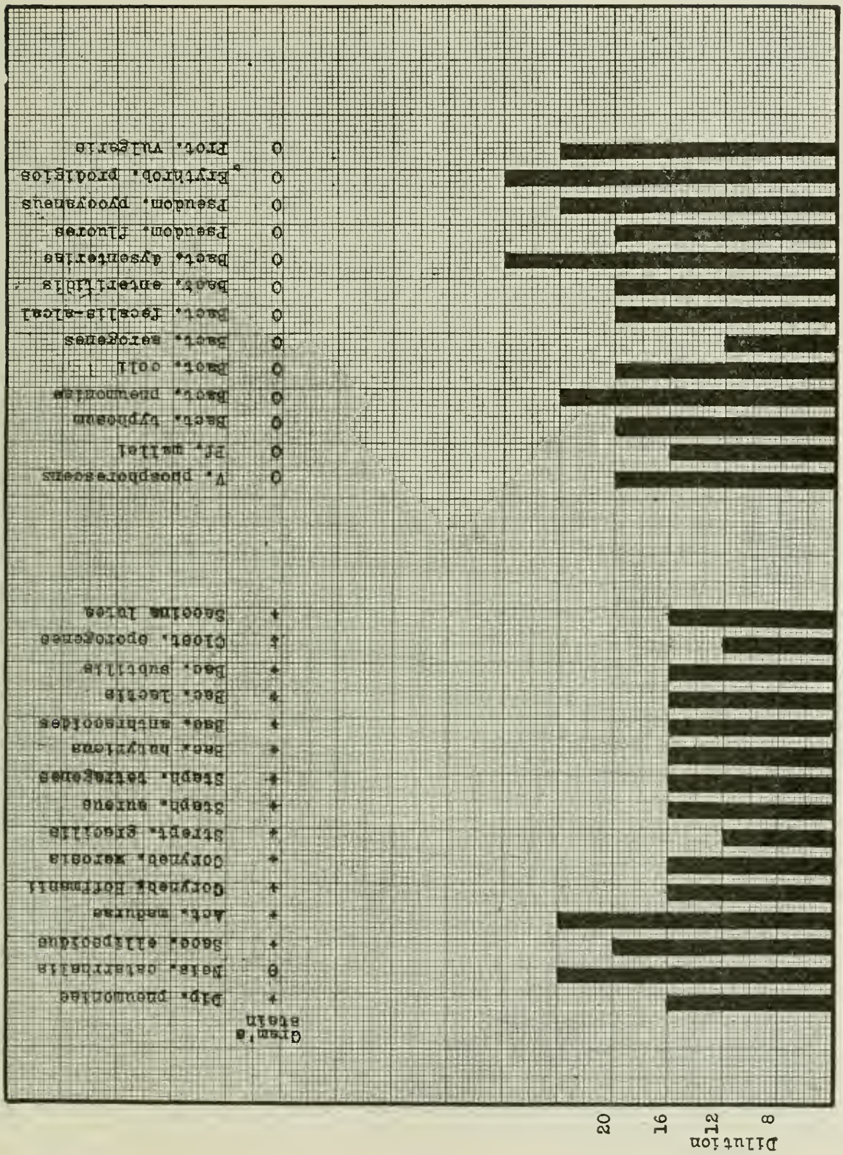

Sensitivity to Mercury Bichloride.
Gram's stain
Prot. vulgaris 0
Erythrob. prodigios 0
Pseudom. pyogeneus 0
Bact. dysenteriae 0
Bact. faecalis-alcal 0
Bact. aerogenes 0
Bact. coli 0
Bact. pneumoniae 0
Bact. typhosum 0
Pf. mallei 0
Sarcina lutea +
Clost. sporogenes +
Bac. anthracoides +
Bac. butyricus +
Staph. tetragenus +
Staph. aureus +
Corynb. hoffmanii +
Act. madurae +
Bacc. allisepticus +
Neis. catarrhalis 0
2048
1024
512
256
128
64
32
Dilution x 10-3

CHART V.



Sensitivity to Antimonyl Potassium Tartrate.

CHART VI.



Sensitivity to Ethyl Alcohol.

CHART VII.

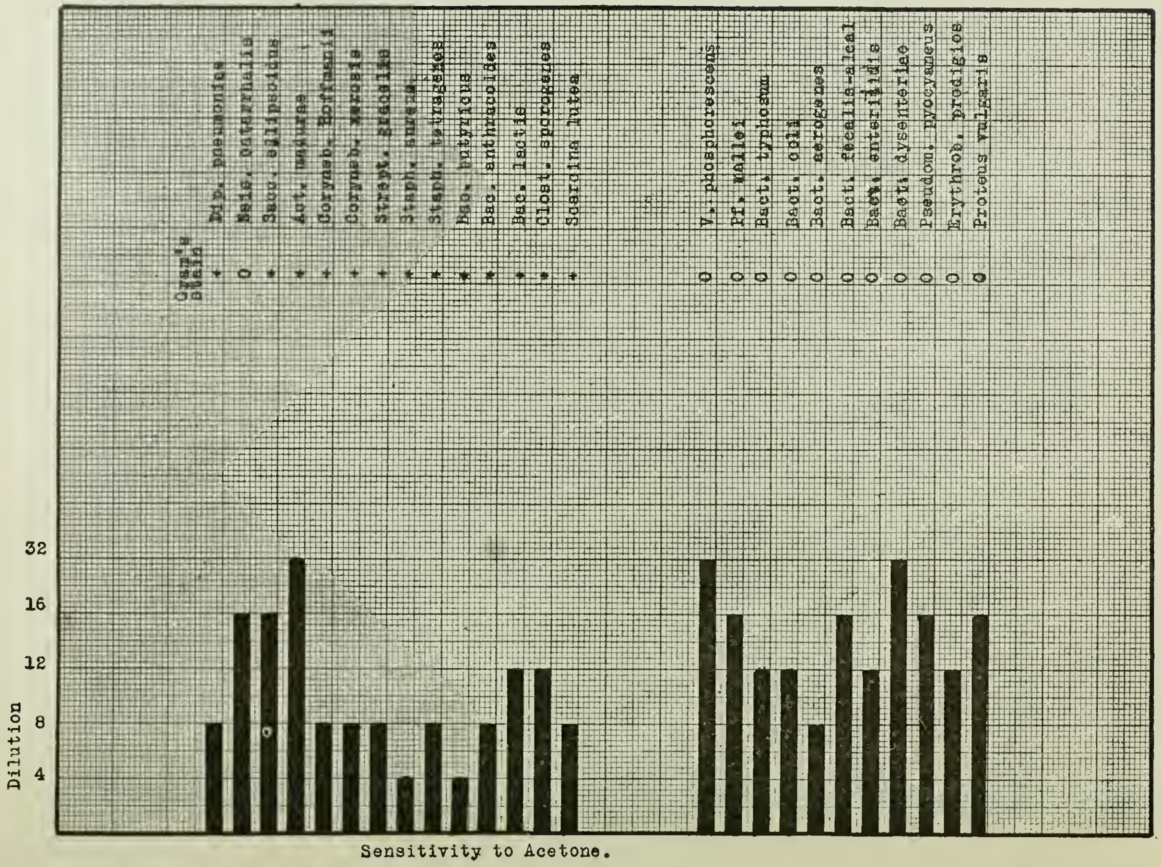

Gram's stain
+ Dip. pneumoniae
0 Neis. catarrhalis
+ Sacc. ellipsoidus
+ Act. madurae
+ Coryneb. Hoffmanii
+ Coryneb. xerosis
+ Strept. fecalis
+ Staph. aureus
+ Staph. tetragenes
+ Bac. butyricus
+ Bac. anthracoides
+ Bac. lactis
+ Clost. sporogenes
+ Sarcina lutea
0 V. phosphorescens
0 Pf. mallei
0 Bact. typhosum
0 Bact. coli
0 Bact. aerogenes
0 Bact. fecalis-alcal
0 Bact. enteritidis
0 Bact. dysenteriae
0 Pseudom. pyocyaneus
0 Erythrob. prodigios
0 Proteus vulgaris
Dilution
32
16
12
8
4
Sensitivity to Acetone.

CHART VIII.

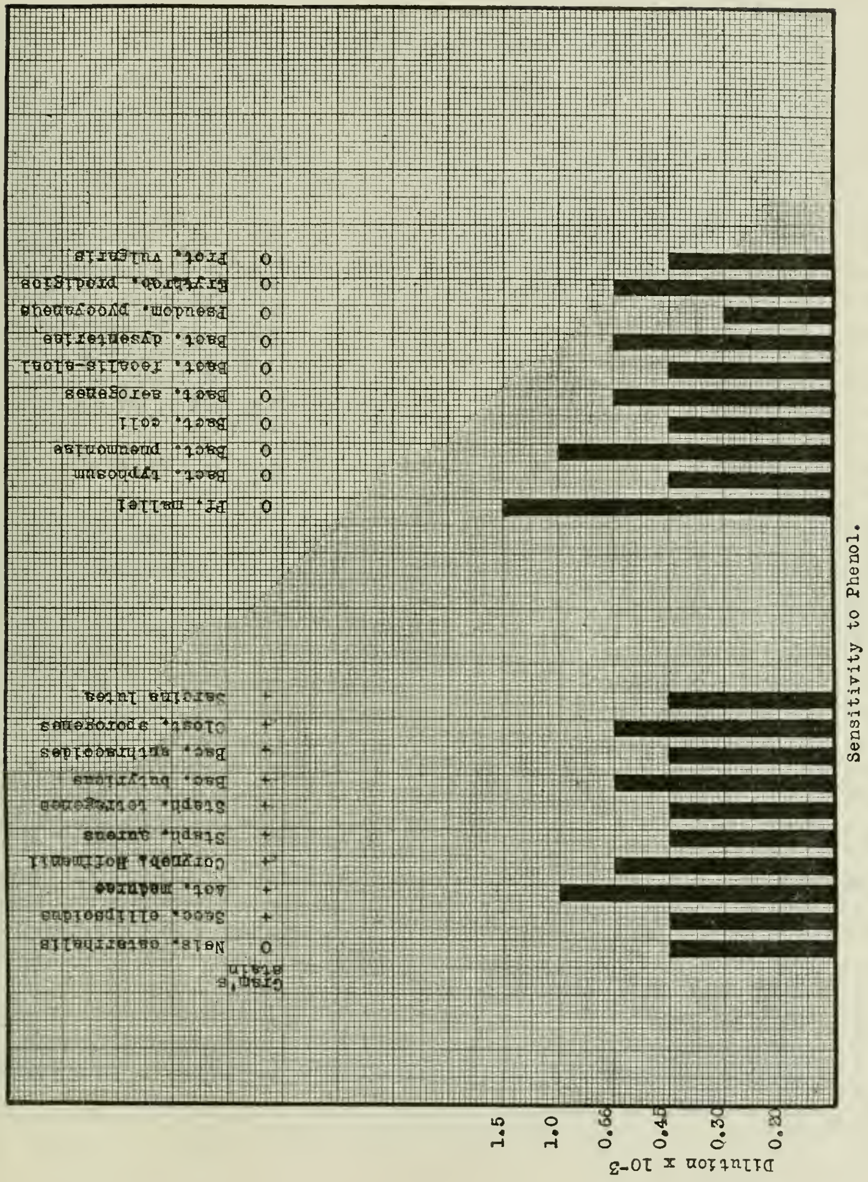
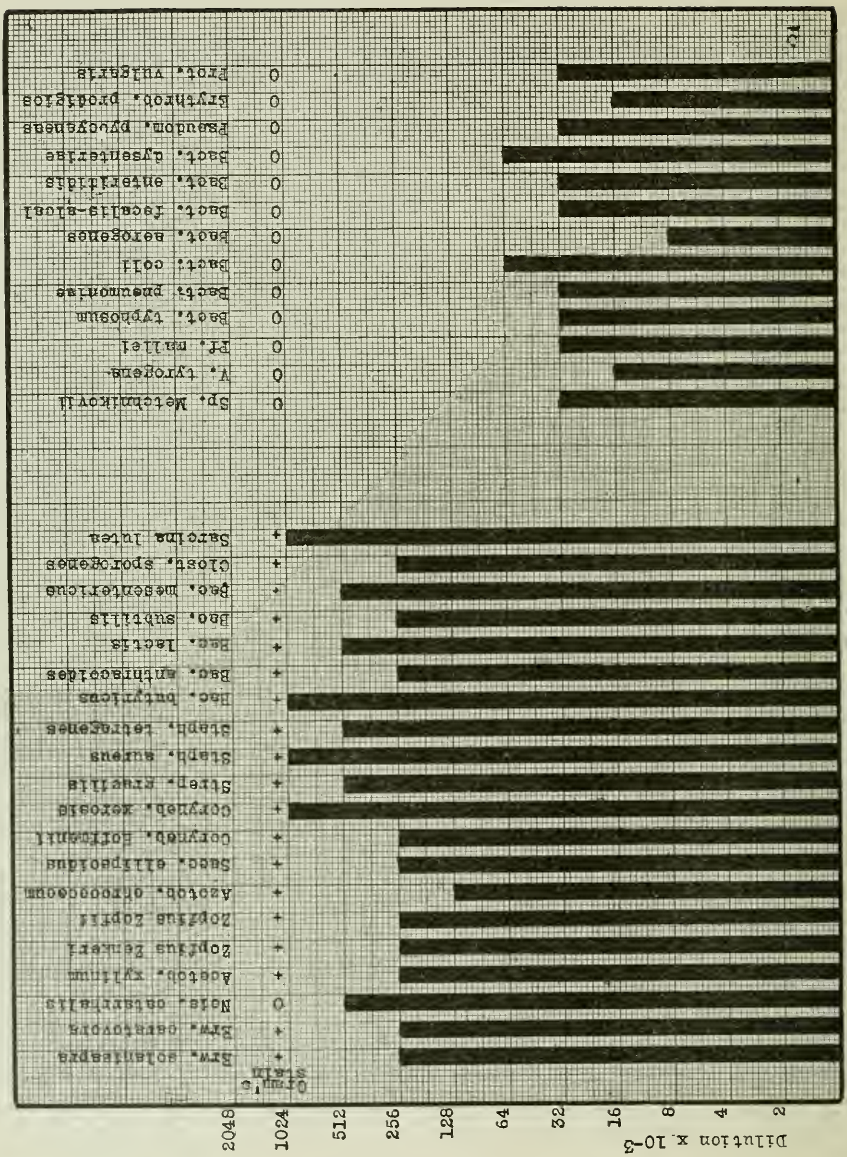

Gram's stain
0 Prot. vulgaris
0 Erythrob. prodigios
0 Pseudom. pyocyanea
0 Bact. dysenteriae
0 Bact. faecalis-alcal
0 Bact. aerogenes
0 Bact. coli
0 Bact. pneumoniae
0 Bact. typhosum
0 Pf. mallei
+ Sarcina lutea
+ Clost. sporogenes
+ Bac. anthracoides
+ Bac. butyricus
+ Staph. tetragenus
+ Staph. aureus
+ Coryneb. Hoffmanii
+ Act. madurae
+ Sacc. ellipsoidea
0 Neis. catarrhalis
1.5
1.0
0.66
0.45
0.30
0.20
Dilution x 10-3
Sensitivity to Phenol.

CHART IX.



Sensitivity to Crystal Violet.

CHART X.

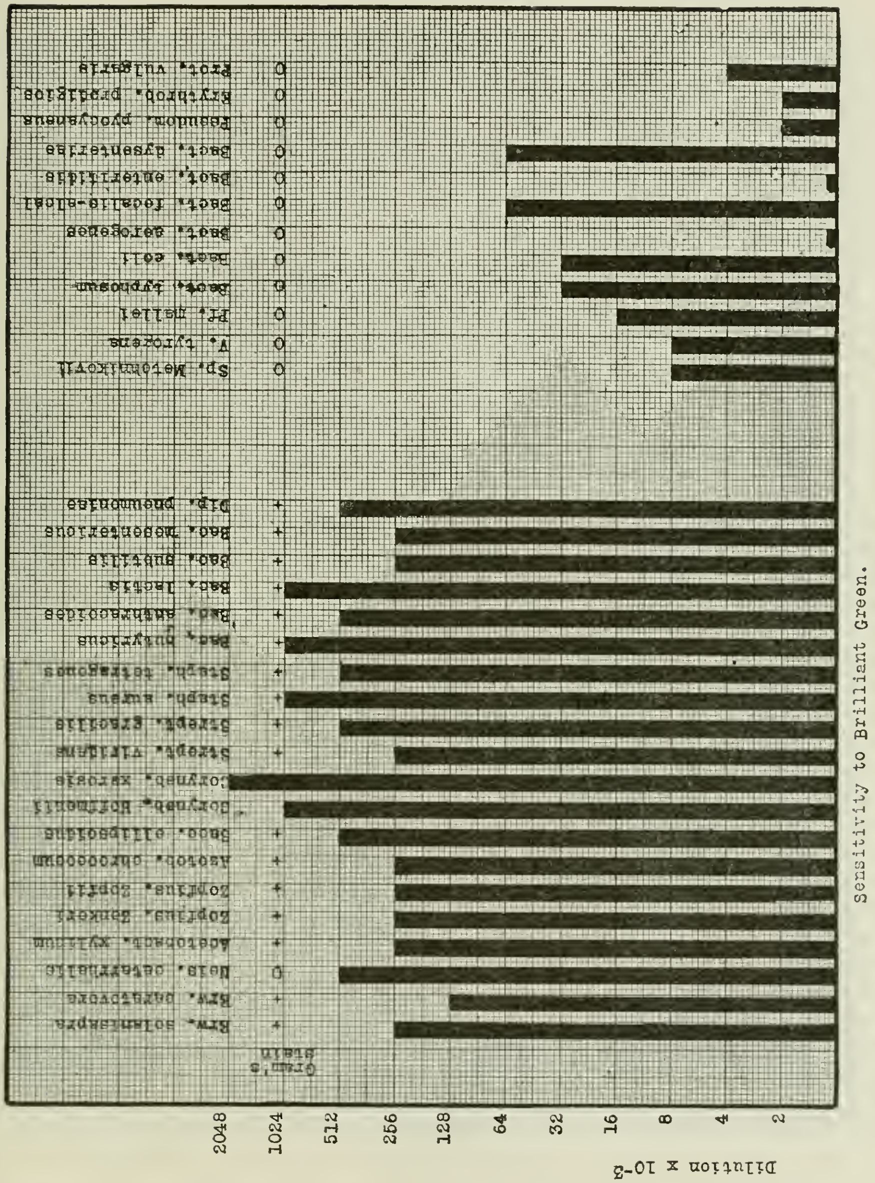
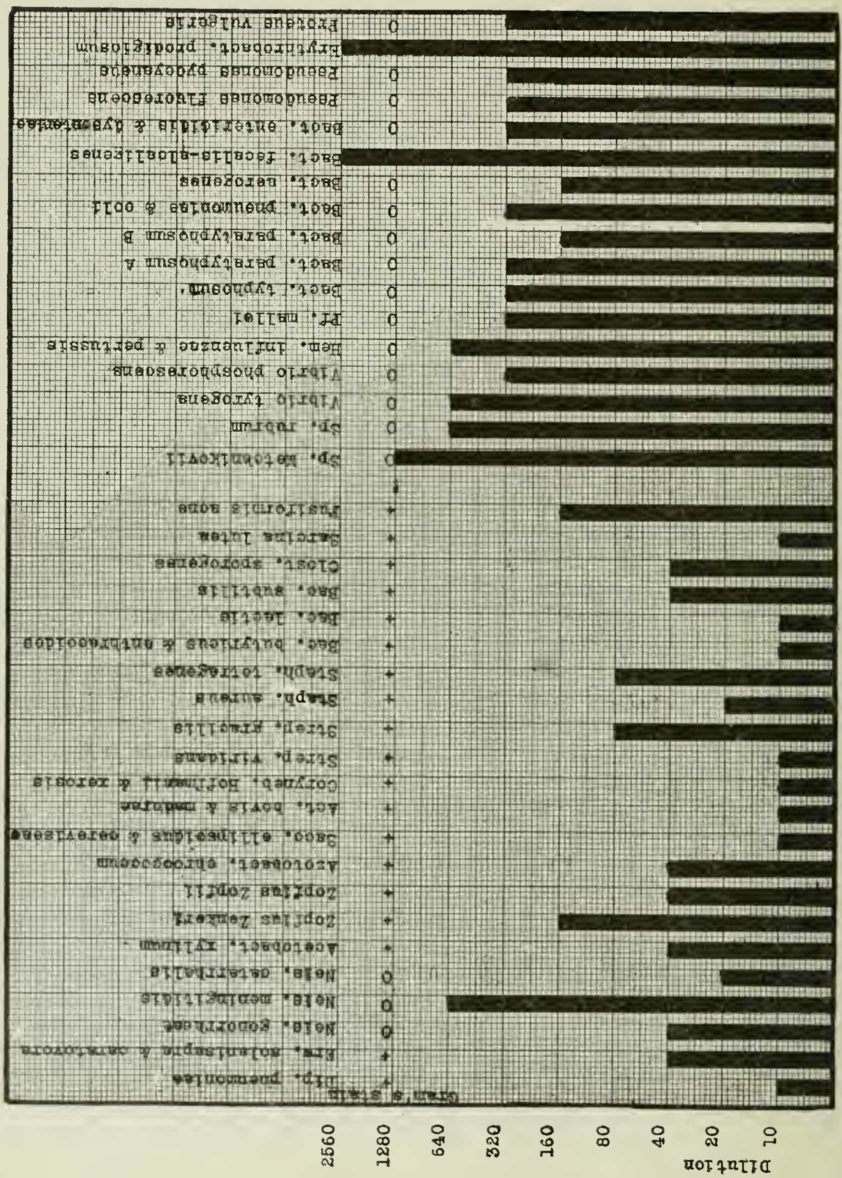
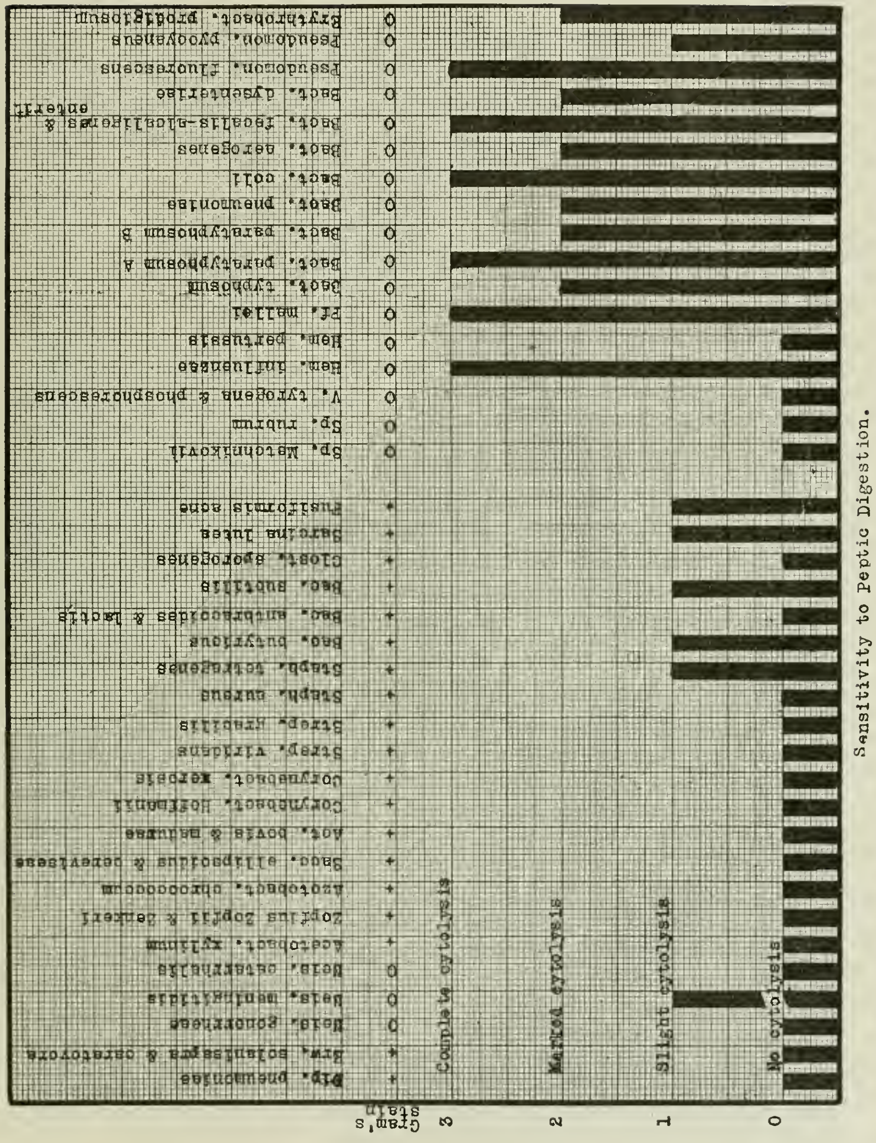

Gram's stain
Erw. solanisapra +
Erw. carotovora +
Neis. catarrhalis 0
Acetobact. xylinum +
Zopfius. Zenkeri +
Zopfius. Zopfii +
Azotob. chroococcum +
Bacc. ellipsoidus +
Coryneb. Hoffmanii
Coryneb. xerosis
Strept. viridans +
Strept. gracilis +
Staph. aureus +
Staph. tetragenus +
Bac. butyricus +
Bac. anthracoides +
Bac. lactis +
Bac. subtilis +
Bac. mesentericus +
Dip. pneumoniae +
Sp. Metchnikovii 0
V. tyrogena 0
Pf. mallei 0
Bact. typhosum 0
Bact. coli 0
Bact. aerogenes 0
Bact. fecalis-alcal 0
Bact. enteritidis 0
Bact. dysenteriae 0
Pseudom. pyocyaneus 0
Erythrob. prodigios 0
Prot. vulgaris 0
2048
1024
512
256
128
64
32
16
8
4
2
Dilution x 10-3
Sensitivity to Brilliant Green.

CHART XI.



Sensitivity to NaOH cytolysis.

CHART XII.


Sensitivity to Peptic Digestion.
Gram's stain
3
2
1
0
Complete cytolysis
Marked cytolysis
Slight cytolysis
No cytolysis
Erythrobact. prodigiosum 0
Pseudomon. pyocyaneus 0
Pseudomon. fluorescens 0
Bact. dysenteriae 0
Bact. fecalis-alcaligenes & enteritidis 0
Bact. aerogenes 0
Bact. coli 0
Bact. pneumoniae 0
Bact. paratyphosum B 0
Bact. paratyphosum A 0
Bact. typhosum 0
Pf. melitensis 0
Hem. pertussis 0
Hem. influenzae 0
V. tyrogena & phosphorescens 0
Sp. rubrum 0
Sp. Metchnikovii 0
Fusiformis acne +
Sarcina lutea +
Clost. sporogenes +
Bac. subtilis +
Bac. anthracoides & lactis +
Bac. butyricus +
Staph. tetragenes +
Staph. aureus +
Strep. fecalis +
Strep. viridans +
Corynebact. xerosis +
Corynebact. Hoffmanii +
Act. bovis & madurae +
Sacc. ellipsoidus & cerevisiae +
Azotobact. chroococcum +
Zopfius Zopfii & Zenkeri +
Acetobact. xylinum +
Neis. catarrhalis 0
Neis. meningitidis 0
Neis. gonorrhoeae 0
Erw. solanisapra & carotovora +
Dip. pneumoniae +

THE EFFECT OF A FOLLOWING INJECTION OF DIPHTHERIA ANTITOXIN ON THE INTRACUTANEOUS TEST FOR THE VIRULENCE OF DIPHTHERIA BACILLI.*

By MARGARET BEATTIE, Gr. P. H.

Instructor in Public Health, University of California.

(Received for publication July 20, 1922.)

In a recent communication describing an intracutaneous virulence test with field cultures containing morphologic diphtheria bacilli, Force and Beattie (1) call attention to the fact that the test has occasionally been invalidated by the death of the inoculated animal before the reaction could be determined. However carefully the cultures are distributed among the guinea pigs, death will occur whenever the toxin-producing organisms are too numerous. If the test is conducted with pure cultures, the inoculum may be diluted in order to avoid an overdose of toxin, but when field cultures are used it is not desirable to dilute the culture suspension, as it is not possible to know the proportion of diphtheria bacilli present.

The death of the animal would be avoided if some method could be devised for neutralizing the toxin after it has combined with the cells at the site of inoculation, but before its effect has become generalized. Consequently the local production of toxin, as it affects the skin, could be observed while the death of the test animal could be prevented, or at least delayed, until the skin lesions were sufficiently advanced to allow the result to be determined.

Recently Eagleton and Baxter (2) have published a note on the intracutaneous virulence test, as affected by what they term the "following dose" of antitoxin. They found that it was possible to prevent the death of the test animal, without interfering with the determination of virulence, by the use of an intraperitoneal injection of 125 units of antitoxin five hours after the inoculation of pure cultures of morphologic diphtheria bacilli.

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Aided by a grant from the Research Board, University of California.

Novotny and Schick (3) found that the cutaneous reaction to diphtheria toxin was markedly influenced by an antitoxin injection given after the inoculation. By inoculating concentrated toxin, according to the drill method of Pirquet, they produced a lesion which reached its maximum development on the third day, at which time it presented an extensive area of inflammation with a central area of necrosis. The character of this reaction was not altered by 200 units of antitoxin administered 24 hours after the inoculation, but the lesion was decidedly modified when the antitoxin was injected five hours after the test inoculation. The maximum development in this latter case was attained in thirty hours. The area of inflammation was not so extensive, but a characteristic area of necrosis was observed. This modified reaction healed more quickly. The authors concluded that the cutaneous reaction was not prevented by the administration of antitoxin five hours after the test inoculation, though its development was decidedly accelerated.

Roemer (4) found that a definite reaction could be observed following the intracutaneous inoculation of 1/500 M.L.D. of diphtheria toxin. However he did not study the effect of a following injection of antitoxin upon these reactions.

In order to determine what effect a "following dose" of antitoxin might have upon the intracutaneous reaction to field cultures containing morphologic diphtheria bacilli, the following experimental work was projected: (1) Two hundred units of antitoxin were administered at varying intervals before and after an intracutaneous inoculation of a fixed amount of diphtheria toxin. (2) Two hundred units of antitoxin were administered five hours after the intracutaneous inoculation of varying amounts of toxin. (3) Two hundred units of antitoxin were administered five hours after the intracutaneous inoculation of pure cultures of virulent diphtheria bacilli. (4) Either 200 or 100 units of antitoxin were administered five hours after the intracutaneous inoculation of field cultures containing morphologic diphtheria bacilli.

In all tests 0.2 c.c. of toxin or of culture suspension was inoculated intracutaneously. The culture suspensions were equalized by comparison with a turbidity standard prepared by suspending in 10 c.c. of salt solution a 24-hour culture of B. typhosus on 10 c.c. of plain agar which had been placed while melted in a standard test tube and solidified in a 10 per cent. slope. The inoculations were made with a 26-gauge needle and a Luer tuberculin syringe. Four to eight tests were placed on the shaven sides of white-skinned guinea pigs. The following injection of antitoxin was administered intraperitoneally five hours after the test inoculation. All tests with field cultures were controlled by being placed on animals which had been given 500–800 units of diphtheria antitoxin subcutaneously at the time the cultures were inoculated. The reactions were measured and recorded for four days, after which the final results were expressed in degrees of necrosis. The typical lesion of advanced necrosis due to diphtheria toxin is an inflammation followed by a coagulative necrosis which is white at first, but becomes purple as the congestion increases. Liquefaction which begins in the center of the area of coagulative necrosis extends, as indicated by fibrous exudate, until this exudate finally covers the entire area of coagulative necrosis as a black crust. This has been designated in the tables as a first degree necrosis (N1). The lesion designated as second degree necrosis (N2) has a circumscribed area of inflammation which increases gradually in size and has a white area of coagulative necrosis in its center. Liquefaction occurs and extends in the area of necrosis, but since it does not involve such deep layers of tissue the exudate is light brown in color. Sometimes there is but a slight area of coagulative necrosis and the liquefaction of tissue does not occur except in scattered punctate areas where the fibrous exudate leaves small brown crusts. In third degree necrosis (N3) the inflammation is succeeded by a necrosis involving the superficial layers of the skin, so that only scaling and brownish pigmentation are noted.

1. Antitoxin Administered Before and After a Fixed Amount of Toxin.

The experiment of Novotny and Schick was repeated substituting the intracutaneous inoculation of 1/10 M.L.D. of diphtheria toxin for "vaccination" with a "ten times concentrated toxin." This amount of toxin was selected, because it gave a definite necrosis without causing the death of the guinea pig, even when eight tests were placed on one animal. The toxin was administered at intervals before and after the intraperitoneal injection of 200 units of antitoxin. (Table I.) When antitoxin was administered 12 or 24 hours after the toxin, the lesion was not modified in any way. When the toxin inoculation preceded the antitoxin injection by five hours the lesion seemed modified on the first day, but terminated in a first degree necrosis of approximately the same size and character as those resulting from the previously placed tests. In one case, where the antitoxin injection followed the toxin inoculation after three hours, the lesion terminated in a first degree necrosis, but in the other, second degree necrosis was observed. The effect of antitoxin administered simultaneously with toxin was still more marked, the site of inoculation becoming only slightly inflamed and no necrosis being observed. The reaction was completely inhibited when the antitoxin was injected 12 hours before the toxin. It would appear from this group of reactions that 200 units of antitoxin, administered five hours after the inoculation of 1/10 M.L.D. of toxin, failed to exert any marked effect on the toxin lesion.

TABLE I.

The effect of two hundred units of diphtheria antitoxin on the reaction produced by the intracutaneous injection of toxin administered at various intervals before and after the antitoxin injection.

Toxin injection.	Time A.T. injection.	Days.			
		1	2	3	4
0.2 c.c. (1/10 M.L.D.)	24 hr. P. I.	(32) 14w	s19s 10 N1	s15) 10 N1	15 N1
	12 hr. P. I.	(24) 12w	s20s 15w 11p	10 N1	14 N1
	5 hr. P. I.	(7)	(16) 15p	s14) 10 N1	11 N1
	3 hr. P. I.	(17)	(17) 8 N1	s10s 9 N1	10 N1
	0 hr.	(13s	s10s	s 9s	s 7s
	2 hr. A. I.	0	0	0	0

Toxin injection.	Time A.T. injection.	Days.			
		1	2	3	4
0.2 c.c. (1/10 M.L.D.)	24 hr. P. I.	(22) 14w 16p	(19) 15w 12p	s17s 12 N1	s18s 10 N1
	12 hr. P. I.	(16s 9w	(18) 11w 8p	(14s 10 N1	s15s 9 N1
	5 hr. P. I.	(8)	(15) 11w 7p	(15) 10 N1	s16s 11 N1
	3 hr. P. I.	(19)	(11)	(11) 8 N2	s19s 13 N2
	0 hr.	(6)	(7)	s 6s	(4s
	2 hr. A. I.	0	0	0	0

P. I. — Post inoculation.
A. I. — Ante inoculation.
Numerals refer to diameter of lesions in mm.
(— induration.
s — before numerals, slight induration.
) — redness.
s — after numerals, slight redness.
p — purple.
w — white.
N1 — 1st degree necrosis.
N2 — 2d degree necrosis.
N3 — 3d degree necrosis.

2. Antitoxin Administered Five Hours After Varying Amounts of Toxin.

It is conceivable that a following injection of antitoxin might affect the lesions produced by amounts of toxin, less than 1/10 M.L.D., either by preventing the characteristic necrosis or by inhibiting the reaction entirely. In order to test this possibility a series of experiments with varying amounts of toxin was performed.

Guinea pigs were inoculated intracutaneously with amounts of toxin ranging from 1/10 to 1/800 M.L.D. Half the animals received 200 units of antitoxin intraperitoneally five hours after the toxin inoculations. The results of these experiments are summarized in Table II. First degree necrosis was observed twice as often on the animals which received no following injection as on the animals which received antitoxin, whereas this relation was practically reversed for third degree necrosis. It will be seen that necrosis occurred in 60 tests made with amounts of toxin ranging from 1/10 to 1/80 M.L.D., inclusive. This necrosis was of the first or second degree in 29 of 30 tests on animals which had no antitoxin, but was of equal severity in only 18 of 30 tests on animals which received the following injection of antitoxin.

TABLE II.

The effect of two hundred units of diphtheria antitoxin on the reaction produced by the intracutaneous injection of varying dilutions of diphtheria toxin.

Toxin dilution.	Degree of necrosis.							
	1st.		2d.		3d.		No reaction.	
	N.	F. A.	N.	F. A.	N.	F. A.	N.	F. A.
1/10 M.L.D.	7	7						
1/20 M.L.D.	5	1		4				
1/40 M.L.D.	2		5	3		4		
1/50 M.L.D.	1		4	2	1	4		
1/80 M.L.D.			5	1		4		
1/100 M.L.D.					3	4	2	1
1/160 M.L.D.					2	3	3	2
1/200 M.L.D.					2		3	5
1/320 M.L.D.					3	1	2	4
1/400 M.L.D.							6	6
1/640 M.L.D.							5	5
1/800 M.L.D.							6	6
Total	15	8	14	10	11	20	27	29

N. — Animal receiving no antitoxin.
F. A. — Animal receiving following injection of antitoxin.
Numerals refer to number of tests.

From these experiments it is evident that the administration of 200 units of antitoxin after the injection of 1/10 M.L.D. of toxin did not affect the toxin lesion. The lesions produced by 1/20 M.L.D. of toxin were slightly modified by the antitoxin, but not enough to interfere with their interpretation. The lesions, produced by amounts of toxin ranging from 1/40 to 1/80 M.L.D., inclusive, were considerably modified by the following injection of antitoxin. With less than 1/80 M.L.D. the reactions were slight (third degree necrosis) or absent on both animals.

TABLE III.

The effect of an injection of two hundred units of diphtheria antitoxin following the intracutaneous inoculation of pure cultures of virulent diphtheria bacilli.

Culture Number.		Days.			
		1	2	3	4
687	N.	s10s	L. E.		
		3p			
	F. A.	(13)	(11)	(8)	
			7p	7 N1	9 N1
236 (2)	N.	s 6s	L. E.		
		3p			
	F. A.	(15)	(14)	s12s	
		6w			
		4p	6p	7 N1	7 N1
236	N.	s10s	L. E.		
	F. A.	(16)	(14)	s14s	
		6w			
		3p	8p	9 N1	10 N1
536 (2)	N.	s 5s	L. E.		
	F. A.	(13)	(13)	s 6s	
		7w			
		3p	9p	4 N1	6 N1

Culture Number.		Days.			
		1	2	3	4
1216..........	N.	(23) 6w 3p	L. E.		
	F. A.	(18) 6w 2p	(13) 10w 6p	(12) 8w 5 N1	s12s 5 N1
536 (1)........	N.	(17) 3p	L. E.		
	F. A.	(9) 6w 3p	(9) 7w 4p	(9) 5 N1	(9s 5 N1
238 (2)........	N.	L. E.			
	F. A.	(19) 8w 5p	(18) 7w 4p	(16) 5w 3 N1	s10s 5 N1
1213..........	N.	L. E.			
	F. A.	(20) 8w 3p	s17) 9w 4p	(10) 4 N1	s10s 4 N1

L. E. — Guinea pig dead.
Other symbols as in Tables I and II.

3. Antitoxin Administered Five Hours After Pure Cultures.

Eight pure cultures of virulent *B. diphtheriae* were inoculated intracutaneously into guinea pigs, half of them receiving 200 units of antitoxin five hours later (Table III). In every case the animals which did not receive antitoxin died of diphtheria toxemia, before the 48-hour measurements could be made, while all the animals which received antitoxin survived. Comparative measurements were consequently impossible, but in each instance the lesions on the animals which received antitoxin were characterized by first degree necrosis.

A comparison of these lesions with those produced by the same cultures, in tests conducted one year previously, shows that the following injection of antitoxin did not modify the local reaction produced by the toxin, produced by pure cultures of *B. diphtheriae.* It is evident that the amount of toxin produced by pure cultures of *B. diphtheriae* is in such excess that the local reaction is not influenced by the following injection of antitoxin. These findings confirm the observations of Eagleton and Baxter.

4. ANTITOXIN ADMINISTERED FIVE HOURS AFTER FIELD CULTURES.

Fifty-seven cultures containing morphologic diphtheria bacilli were obtained from the State Hygienic Laboratory, and each tested for virulence on three guinea pigs, by the intracutaneous method. The first animal received no antitoxin, the second either 100 or 200 units of antitoxin five hours after the culture inoculation, and the third received 500 units of antitoxin at the time of the culture inoculation.

TABLE IV.

The effect of an injection of diphtheria antitoxin following the intracutaneous inoculation of field cultures containing morphologic diphtheria bacilli.

Result.	Virulence.	Group 1.		Group 2.	
		N.	F. A. 200 units.	N.	F. A. 100 units.
Death.........	Undetermined	9	0	16	4
Death.........	Positive	10	5	4	6
Death.........	Negative	5	2	1	2
Survival.......	Positive	5	12	0	9
Survival.......	Negative	7	17	0	0
Total.........		36	36	21	21

The results of these tests are summarized in Table IV. The first group consists of 36 cultures tested with and without a following injection of 200 units of antitoxin. The second group consists of 21 cultures similarly tested, the following injection, in this case, being 100 units of antitoxin. It will be noted that the unprotected animals, on which 24 of the first group of 36 cultures were placed, succumbed, the diagnosis of virulence being impossible in nine instances.

On the other hand, only seven of the same 36 cultures, placed on animals receiving 200 units of antitoxin, were on animals which succumbed, and a diagnosis of virulence was possible for all of these seven cultures.

All 21 of the cultures in the second group were on unprotected animals which succumbed, and in 16 instances a diagnosis of virulence was impossible. Twelve of the 21 cultures on animals receiving 100 units of antitoxin were on animals which succumbed, and a diagnosis of virulence was impossible for four of these twelve. This failure to make a diagnosis was not due, however, to absence of antitoxin protection of the animals receiving three of the four cultures, since death did not occur until the fourth day after the inoculation. The failure of diagnosis was due to a contaminating organism which produced an atypical appearance of the local lesions resulting from the inoculation of these three cultures. The exclusion of these three cultures leaves nine on animals which succumbed in spite of 100 units of antitoxin. The percentage distribution of cultures on animals which succumbed was therefore as follows:

Group 1 (following injection of 200 units):

24 of 36 (67 per cent.) on unprotected animals.
7 of 36 (19 per cent.) on protected animals.

Group 2 (following injection of 100 units):

21 of 21 (100 per cent.) on unprotected animals.
9 of 21 (43 per cent.) on protected animals.

It is apparent that the administration of antitoxin five hours after the intracutaneous virulence test with field cultures either preserves the life of the test animal, or at least prolongs it until the determination of virulence is assured. It is also apparent that a following injection of 200 units of antitoxin exerts a greater protective influence than a following injection of 100 units of antitoxin, though this greater influence apparently does not affect the character of the local lesions.

One culture which produced a first degree necrosis on the unprotected animal produced no necrosis on the animal which had received 200 units of antitoxin as a following injection. This was the only instance in which the following injection interfered with the true diagnosis of virulence. On the other hand, the diagnosis of 25 cultures would have remained undetermined, on account of the death of the unprotected animals, had they not also been inoculated on animals receiving the following injection of antitoxin.

SUMMARY.

I. The intraperitoneal administration of 200 units of antitoxin, simultaneously with the intracutaneous injection of 1/10 M.L.D. of diphtheria toxin, inhibited the local toxin lesions. The administration of 200 units of antitoxin, five hours after the intracutaneous injection of 1/10 M.L.D. of toxin, did not influence the toxin lesion.

II. The administration of 200 units of antitoxin in five hours after the intracutaneous injection of 1/20 M.L.D. of diphtheria toxin slightly modified the local toxin lesion, but not enough to interfere with its interpretation. Lesions produced by 1/40 to 1/80 M.L.D. were considerably modified by the following injection of antitoxin.

III. The administration of 200 units of antitoxin, five hours after the intracutaneous inoculation of pure cultures of virulent diphtheria bacilli, did not influence the local toxin lesions, but prevented the death of all animals subject to the tests. This confirms the observation of Eagleton and Baxter.

IV. The administration of 200 units of antitoxin, five hours after the intracutaneous inoculation of field cultures containing morphologic diphtheria bacilli, did not influence the local toxin lesions, but prevented the death of the inoculated animals, or at least preserved them until the determination of virulence was assured.

CONCLUSIONS.

Since the intracutaneous lesion produced by as small an amount of diphtheria toxin as 1/20 M.L.D. is not inhibited by the administration of an intraperitoneal injection of 200 units of antitoxin five hours after the injection of the toxin, it follows that a protective dose of antitoxin can be administered not only after intracutaneous virulence tests with pure cultures of morphologic diphtheria bacilli, as suggested by Eagleton and Baxter, but also can be administered after intracutaneous virulence tests with field cultures. This is possible because practically all virulent field cultures contain enough diphtheria bacilli to produce at least 1/20 M.L.D. of toxin, when inoculated intracutaneously according to the technique described by Force and Beattie.

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THE RELATION BETWEEN BREAST AND ARTIFICIAL FEEDING AND INFANT MORTALITY.*

By ROBERT M. WOODBURY, Ph.D.,

Director, Statistical Research, Children's Bureau, U. S. Department of Labor.

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The superiority of breast over artificial feeding during the early months of infancy has long been recognized by the medical profession, and general principles in regard to the best modes of infant feeding have been developed. In view of the importance of the subject, however, statistical evidence of the relative influence of artificial and breast feeding upon infant mortality rates seems surprisingly meagre. Most of the data which are available relate to foreign cities or districts where special censuses of the kind of feeding received by the infant population have been taken, and used in connection with records of the kind of feeding received by infants who died, or where other types of special inquiries have been made to ascertain the type of feeding prevailing.†

In explanation of the relative lack of material on this subject, it should be mentioned that sound statistical treatment requires a comparison of the deaths of infants receiving each type of feeding with the time lived by infants while receiving the same type of feeding,—information which is not readily available.

In the course of studies of infant mortality made in eight American cities: Johnstown, Pa., Manchester, N. H., Saginaw, Mich., New

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* Summary of a chapter on the relation between infant feeding and infant mortality, to be published later in a bulletin of the Children's Bureau.

† For example, Westergaard, H., in "Die Lehre von der Mortalität und der Morbilität," Revised Edition, 1901, p. 361, gives figures secured by Böckh for Berlin, based on a census of infants in the city and the returns as to type of feeding contained on death certificates. Dr. Groth and Professor Hahn made an extensive survey of the communes in Bavaria, in which the type of feeding was ascertained for samples of infants brought to vaccination stations (Die Säuglingsverhältnisse in Bayern, Zeitsch. d. k. Bayerisch-Statistischen Landesamts, 42d year, 1910, No. 1, pp. 78–164). For a statistical study of feeding in Boston, Mass., based upon a sample group of infants, see Davis, William H., M.D., Statistical comparison of the mortality of breast-fed and bottle-fed infants, Amer. Jour. Diseases of Children, V, 1913, pp. 234–247.

Bedford, Mass., Brockton, Mass., Waterbury, Conn., Akron, Ohio, and Baltimore, Md.,—the Children's Bureau obtained information for a large number of infants, including in each case a record of the feeding received in each month of life, from birth until the first birthday. The following analysis of the influence of feeding is based upon data for 22,422 live born infants in these eight cities.*

These 22,422 infants are classified according to the feeding received during each month of the first year of life.† The classification by months not only yields a statement of the total number of months lived by infants receiving each type of feeding, but also permits taking into account the change in the mortality rate by age, and comparing for each month during the first year of life the relative mortality among infants receiving each type of feeding.

The kinds of feeding are classified into the three groups, exclusively breast, exclusively artificial, and partly breast and partly artificial feeding. This grouping is not wholly satisfactory. The groups of partial and exclusive artificial feeding are not classified in detail according to the character of the food given, and hence the analysis of the effect of feeding on infant mortality made in the present study is confined to the differences between exclusively breast feeding and exclusively artificial feeding, and between partial breast feeding and each of the other two groups, irrespective of the character of the artificial feeding.

Table I shows the increase in prevalence of artificial feeding from month to month during the first year of life. In the first month 86.7 per cent. of the infants were breast fed, and 3.1 per cent. were partly breast fed, while 10.2 per cent. were exclusively artificially fed. In the third month the proportion exclusively breast fed had fallen to 71.4, while the proportion artificially fed had doubled, reaching 20.4 per cent. By the sixth month the proportion exclusively breast fed was only slightly over half, while that artificially fed had risen to nearly three tenths. By the ninth month less than three tenths, 28.1 per cent., were exclusively breast fed, while the

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* This number excludes from consideration 545 infants, or 2.4 per cent. of the total number, who died not fed.

† If the feeding changed during the month the infant was classified according to the type he received during the greater part of the month, since this rule gives the closest approximation to the months lived by infants receiving each type of feeding. In case of death during the month, the type of feeding received prior to death (except in cases where the feeding was changed by reason of sickness) was tabulated.

TABLE I.

Type of feeding, by month of life; infants in eight cities.

Month of life.	Infants surviving at beginning of specified month of life.							
	Total.	Exclusively breast fed during the month.		Partly breast fed during the month.		Exclusively artificially fed during the month.		Not reported.
		Number.	Per cent.	Number.	Per cent.	Number.	Per cent.	
First	22,422*	19,438	86.7	686	3.1	2,287	10.2	11
Second	21,939	17,178	78.3	1,294	5.9	3,457	15.8	10
Third	21,735	15,509	71.4	1,786	8.2	4,431	20.4	9
Fourth	21,560	13,516	62.7	2,668	12.4	5,366	24.9	10
Fifth	21,387	12,263	57.3	3,317	15.5	5,797	27.1	10
Sixth	21,222	10,969	51.7	4,098	19.3	6,145	29.0	10
Seventh	21,066	8,641	41.0	5,801	27.5	6,615	31.4	9
Eighth	20,934	7,343	35.1	6,671	31.9	6,911	33.0	9
Ninth	20,812	5,853	28.1	7,684	36.9	7,268	34.9	7
Tenth	20,693	4,248	20.5	8,591	41.5	7,845	37.9	9
Eleventh	20,584	3,278	15.9	9,062	44.0	8,235	40.0	9
Twelfth	20,504	2,736	13.3	9,145	44.6	8,616	42.0	7

* Excluding 545 infants who died not fed.

proportion partly breast fed had increased to 36.9 per cent. and that exclusively artificially fed had increased to 34.9 per cent. At the end of the first year of life, only 13.3 per cent. of the infants continued to be entirely breast fed, but the proportion partly breast fed had risen to 44.6 per cent. and that wholly artificially fed to 42.0 per cent. Up to the ninth month the proportion of artificially fed exceeded that of partly breast-fed infants. From the ninth to the twelfth this relative position is reversed, and the proportion partly breast fed was slightly greater than that exclusively artificially fed. The greatest number of transfers from exclusive breast to partly breast or to artificial feeding took place between the sixth and the seventh months.

These figures can be summed up in convenient fashion in terms of months lived by infants who received each type of feeding. Taking for this purpose the figures for the first nine months only, since, as will be shown later, the type of feeding in these months is of greater importance than in the last three months of the first year of life, it is found that of the 192,212 months lived 57.4 per cent. were lived by infants while breast fed, 17.6 per cent. while partly breast fed, and 24.9 per cent. while artificially fed. Thus not far from three fifths of the months lived up to and including the ninth month were months of breast feeding, and approximately one fourth were months of artificial feeding.

TABLE II.

Monthly mortality rates, by type of feeding; infants in eight cities.

Month of life.	Monthly probability of dying per 1000 infants.			
	All types of feeding.	Exclusively breast-fed.	Partly breast-fed.	Exclusively artificially fed.
First	44.8*	16.9	36.4	54.7
Second	9.3	5.8	14.7	24.6
Third	8.1	3.7	12.9	21.2
Fourth	8.0	3.4	9.0	19.2
Fifth	7.7	3.3	5.7	18.1
Sixth	7.4	2.1	5.9	17.7
Seventh	6.3	1.9	4.0	14.1
Eighth	5.8	2.9	3.3	11.3
Ninth	5.7	3.2	2.9	10.7
Tenth	5.3	3.8	2.3	9.3
Eleventh	3.9	2.4	2.5	6.0
Twelfth	4.5	4.4	2.7	6.4

* The rate per 1000 fed is 21.5; 545 infants died not fed.

In Table II monthly death rates are shown for each month of life up to the twelfth. By comparing the rates for the three types of feeding in each month of life, the relations between the mortality rates are made clear. In the first month the artificially-fed infants had a rate over 3 times that among the breast-fed infants; in the second month it was 4 times, in the third nearly 6 times, in the fourth, fifth, sixth and seventh months over 5 times, in the eighth 4 times, and in the ninth slightly over 3 times the rates for breast-fed infants. After the ninth month the relative advantage of breast feeding is much less.

The relation between the rates for partly breast-fed infants and those for infants in the other two groups is also shown. In each month up to the eighth the death rate for the partly breast-fed was higher than that for the wholly breast-fed, and lower than that for the artificially fed infants. In the first month the rate was twice that for exclusively breast-fed infants; it rose to nearly 3 times in the second month; in the third it was over 3 times, in the fourth nearly 3 times, in the fifth nearly twice, in the sixth nearly 3 times, in the seventh twice, in the eighth slightly greater, while in the ninth it was slightly less than the rate among wholly breast-fed infants. During the first nine months, the rate among partly breast-fed averaged about twice as high as that among wholly breast-fed infants. For the same period the death rate among the exclusively artificially fed averaged about 3 times as high as among the partly breast-fed babies.

A convenient method of summing up the relative disadvantage of artificial feeding shown in Table II is to compare the deaths which actually occurred among the artificially fed infants with the number that would have occurred, if the mortality rates among the breast-fed infants had prevailed among them. In this comparison, furthermore, allowance can be made for the decrease in the mortality rates by age.* Applying the monthly death rates for breast-fed infants to the infants who were artificially fed in the corresponding months, it is found that instead of the 1047 deaths that actually occurred among the artificially fed infants under one year of age only 268.7 deaths would have been expected at these rates of mortality. In other words the actual deaths were nearly four times the expected deaths.

Comparing in a similar way the number of deaths that actually occurred among all the partly breast-fed infants under 1 year with the number that would have occurred among them, if the rates among the breast fed had prevailed, it is found that only 203.9 deaths would have occurred instead of 269. The death rate among the partly breast-fed averaged slightly higher than among the breast-fed infants.

When the comparison is limited to the first nine months of life, however, the excess mortality both among artificially and among partly breast-fed infants appears more marked. Thus, during the first nine months 870 deaths of artificially fed infants occurred, as compared with only 181.3 that would have been expected at the rate of mortality prevailing among breast-fed infants. In other words, during this period the rates of mortality among the artificially fed averaged nearly five times as high as among the breast-fed infants. Among the partly breast-fed infants 201 deaths occurred during the first nine months, as compared with only 109.3 deaths expected on the basis of the rates prevailing among breast-fed babies. The monthly death rates among the partly breast-fed babies averaged, therefore, not quite twice as high as those among the more favored group of infants who received breast feeding.

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* Professor Westergaard's method of expected deaths can be used to measure the influence of a single element, such as type of feeding, with other factors, age, etc., constant. (See R. M. Woodbury, Westergaard's method of expected deaths as applied to the study of infant mortality. Quarterly Publications of the American Statistical Association, Sept. 1922).

The evidence is suggestive which these statistics offer on the subject of the relative advantage or disadvantage of a change from breast feeding to partly breast or to artificial feeding at different times during the first year of life. The contrast in the mortality rates among infants whose breast feeding ceased in a given month with those among infants who continued to be breast fed is shown in Table III in the form of a comparison between the average monthly death rate from each month to the end of the first year among two groups of infants, those whose breast feeding ceased with or was first supplemented during the month, and those whose breast feeding continued to the end of the year.

The average monthly death rates from the second, third, fourth, fifth and sixth months to the end of the first year among infants whose breast feeding ceased or was supplemented, beginning in these months, were higher than the corresponding rates among infants who were exclusively breast fed to the end of the year. When the change from breast to partially or to exclusively artificial feeding took place in the seventh month or afterwards, however, the average monthly death rates were slightly less than the corresponding rates among infants who were breast fed up to the end of the year.

A comparison of these average rates with the monthly rates in the different months, among infants who were breast fed during those months, indicates not only that the breast-fed infants had lower rates in the seventh and eighth months than the average rates from the seventh or eighth months to the end of the year for infants whose type of feeding changed from exclusively breast feeding, but also that they had lower rates than prevailed among exclusively breast-fed infants later in the first year of life.

TABLE III.

Relative advantage or disadvantage of a change from exclusively breast feeding, as shown by average monthly death rates from time of change to end of first year of life, by month of life in which change was made; infants in eight cities.

Month of life.	Infants whose breast feeding ceased or was supplemented beginning with specified month.			Infants exclusively breast fed.	
	Subsequent months of lifetime until end of first year.	Deaths.	Average monthly death rate per 1000.	Average monthly death rate from specified month to end of year.	Monthly death rate in specified month.
First	7,509*	92	12.3	4.5	16.9
Second	19,811	260	13.1	3.3	5.8
Third	14,915	155	10.4	3.1	3.7
Fourth	16,915	111	6.6	3.0	3.4
Fifth	9,480	44	4.6	3.0	3.3
Sixth	8,652	36	4.2	3.0	2.1
Seventh	13,722	36	2.6	3.1	1.9
Eighth	6,357	21	3.3	3.3	2.9
Ninth	5,848	14	2.4	3.4	3.2
Tenth and later	7,190	12	1.7	3.5	3.5

Table IV gives evidence concerning the relative advantage or disadvantage of weaning at different times during the first year of life, by comparing the average monthly death rate from the month of weaning to the end of the first year among infants whose wholly artificial feeding commenced in a given month with the average monthly death rate among breast-fed infants for the same period.*

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* The rate for the first month is not exactly comparable with those for later months. The figures for the second month, for example, include infants who were exclusively breast fed in the first, but either partially or exclusively artificially fed in the second; the figure for the first month, however, includes only infants who were partially breast fed during the greater part of the month, and these may or may not have received exclusively breast feeding for a period not exceeding two weeks. Infants who received exclusively breast feeding for less than 2 weeks and were then transferred to exclusively artificial feeding, on the other hand, are not included. These differences may explain the slightly lower rate found for the first month as compared with the second.

The figures show higher mortality rates among infants who were weaned before the eighth month than among infants who continued to be breast fed. Among infants weaned after the eighth month, however, the mortality rates were not substantially higher than among infants who continued to be breast fed. Unfortunately the cutting off in the material available for study of the period of observation at one year causes the number of months of observation and of deaths to diminish rapidly, with the result that the numbers, especially for changes after the eighth month, are small.

TABLE IV.

Relative advantage or disadvantage of a change to exclusively artificial feeding, as shown by average monthly death rates from time of change to end of first year of life, by month of life in which change was made; infants in eight cities.

Month of life.	Infants whose exclusively artificial feeding commenced in specified month.			Exclusively breast-fed infants.	
	Months of lifetime up to end of year.	Deaths.	Average monthly death rates per 1000.	Average monthly death rate up to end of year.	Monthly death rate in specified month.
First	23,189	535	23.1	4.5	16.9
Second	13,089	196	15.0	3.3	5.8
Third	10,015	128	12.8	3.1	3.7
Fourth	8,896	89	10.0	3.0	3.4
Fifth	4,149	34	8.2	3.0	3.3
Sixth	3,112	27	8.7	3.0	2.1
Seventh	3,425	17	5.0	3.1	1.9
Eighth	1,942	6	3.1	3.3	2.9
Ninth and later . . .	5,059	16	3.2	3.4	3.4

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* In Table IV infants exclusively artificially fed at some time during the first year of life are classified according to the month in which exclusively artificial feeding began; in Table III infants exclusively breast fed at some time during the first year of life are classified according to the month in which breast feeding ceased or ceased to be exclusive.

A comparison of the monthly death rates among all infants, exclusively artificially fed during the ninth to twelfth months, 10.7, 9.3, 6.0, 6.4, as shown in Table II, with the average rate from the time of commencing exclusively artificial feeding up to the end of the year, among infants exclusively artificially fed, beginning in the ninth and following months, 3.4, as shown in Table IV, indicates clearly that artificial feeding in the early months has a cumulative effect which appears in excessive mortality in the later months. Table V, which shows monthly death rates in each month of life for the groups of infants whose artificial feeding commenced in the different months up to the ninth, brings out this effect clearly. In

TABLE V.

Monthly death rates, by duration of previous artificial feeding; infants in eight cities.

Month of life.	Deaths in month per 1,000 infants surviving at beginning of month.		Deaths in month per 1,000 infants surviving at beginning of month, who were artificially fed from—								
	Exclusively breast fed.	Artificially fed.	First month.	Second month.	Third month.	Fourth month.	Fifth month.	Sixth month.	Seventh month.	Eighth month.	Ninth and later.
First......	16.9	55.2*	55.2								
Second....	5.8	24.7	33.6	10.0							
Third.....	3.7	21.3	26.1	21.1	12.2						
Fourth....	3.4	19.2	29.8	19.9	16.1	1.0					
Fifth......	3.3	18.1	24.0	18.7	18.3	13.6	3.8				
Sixth......	2.1	17.8	18.9	18.2	21.6	21.6	9.4	4.4			
Seventh...	1.9	14.1	19.8	19.4	8.0	12.0	11.4	11.0	3.5		
Eighth....	2.9	11.3	9.8	18.1	13.1	14.2	7.7	4.5	8.7	2.6	
Ninth.....	3.2	10.7	14.3	15.8	13.3	11.3	5.8	11.2	1.7	2.6	
Tenth.....	3.8	9.3	14.0	6.2	9.3	10.4	11.7	11.3	12.3	5.1	1.8
Eleventh..	2.4	6.3	6.8	10.8	8.4	3.2	7.9	6.9	3.5	5.2	3.9
Twelfth...	4.4	6.2	13.1	4.5	6.3	2.1	8.0	11.5	—	—	4.0

* The slight differences which appear between these rates and those shown in Table II are due to the omission in this table of a small number of cases in which exclusively artificial feeding was begun, but was later changed to partial or exclusively breast feeding.

each month of life the mortality rate was higher the longer the period of previous artificial feeding. For example, among infants whose artificial feeding commenced in the first month, the rate in the fifth was 24.0, as compared with rates in the fifth month of 18.7 among those whose artificial feeding commenced in the second month, 18.3 in the third, 13.6 in the fourth, and only 3.8 in the fifth.

The comparison is even more striking when the rates among the artificially fed are compared with those among the exclusively breast-fed infants. Thus, the mortality in the fifth month prevailing among infants who had been artificially fed from the first month, or from birth was 7 times as high as among the breast fed; the mortality among infants who had been artificially fed from the second month was nearly 6 times, from the third month over 5 times, and from the fourth month 4 times, as high as among infants who had been breast fed from birth, while the rate among infants whose artificial feeding commenced in the fifth month was only slightly greater than that among breast-fed infants. The rates in other months of life show, in general, similar increases with increase in the length of previous artificial feeding.

The mortality rate among artificially fed infants in any month, therefore, is an average of rates among groups of infants with different durations of artificial and breast feeding. Other things being equal, the longer the period of previous artificial feeding the higher is the rate, and the longer the period of previous breast feeding the lower the rate.

As already shown, the cumulative effect of artificial feeding appears not only in the relatively higher mortality rates in a given month among the infants who have been artificially fed for the longest periods, but also in an increase in the relative mortality in each group, as the duration of artificial feeding increases when compared with that among breast-fed infants. The first mentioned effect is due in part, as will be shown later, to the fact that the groups of infants artificially fed in the first months have more than their due proportions of twins and triplets, among whom the mortality rates are especially high. But the increase in relative mortality within each group, as the duration of artificial feeding increases, which is observed during the first four or five months, following the commencement of early artificial feeding, can be explained only in terms of the cumulative effect of the type of feeding. Among the infants whose exclusive artificial feeding commenced in the second month, for example, a mortality rate prevailed in that month which was 1.7 times the rate among breast-fed infants; the mortality rate in the third month, however, was 5.7 times, and in the fourth month 5.9 times the corresponding rates among breast-fed infants. Among the infants whose exclusively artificial feeding commenced in the third month a rate prevailed in that month which was 3.3 times, but in the fourth month 4.7 times, and in the fifth month 5.5 times the corresponding rates for breast-fed infants. This increase in the differences between the relative rates, as the length of artificial feeding increases, points to the conclusion that the effect of the type of feeding is cumulative.

TABLE VI.

Excess mortality among artificially fed over that among breast-fed infants, by cause of death; infants in eight cities.

Cause of death.	Deaths among artificially fed infants.		
	Actual.	Expected.*	Ratio of actual to expected.
Total	1,047	268.4	3.9
Gastric and intestinal diseases	495	64.2	7.7
Respiratory diseases	185	100.4	1.8
Malformations	26	5.9	4.4
Early infancy	165	27.1	6.1
Epidemic diseases	74	32.7	2.3
External causes	3	2.5	1.2
Diseases, ill defined or unknown	18	3.7	4.9
All other causes	81	31.9	2.5

An analysis of the death rates by cause of death (Table VI) shows, as would be expected, that the excess mortality among infants, who were artificially fed, was greatest from gastric and intestinal diseases, the diseases especially connected with improper feeding. From the second to the ninth months the mortality from this cause among artificially fed infants was 11.3 times as great as among breast-fed infants, while in the tenth, eleventh, and twelfth months the excess mortality dropped rapidly, until it was only 3.5 times as great. During the entire first year of life the mortality from this group of

* Expected, if the rates from each cause that prevailed among breast-fed infants had prevailed among the artificially fed infants.

diseases among artificially fed infants averaged 7.7 times the rate among breast-fed infants. If the rates from gastric and intestinal diseases prevailing among breast-fed infants had prevailed among artificially fed infants, only 64 deaths would have been expected, as compared with the 495 which actually occurred from these causes.

Not only are the rates from gastric and intestinal diseases markedly higher, but the rates from all the other principal causes appear to be higher among artificially fed than among breast-fed infants. Among the artificially fed the death rate from respiratory diseases averaged 85 per cent. higher than among the breast-fed babies. The rates from malformations and from early infancy were four and six times as high respectively. The rate from epidemic diseases was over twice as high. The group of all other causes, including convulsions, meningitis, and congenital heart disease, had a mortality rate two and a half times as high, while the group of ill-defined causes had a rate nearly 5 times as high among artificially fed, as among breast-fed infants.

To a certain extent the higher rates from "all other causes" may be attributed to digestive disorders—in so far as they represent deaths ascribed to convulsions following upon gastric and intestinal diseases. Similarly the group of deaths from ill-defined causes may include many from these diseases. Even the group of causes peculiar to early infancy, especially in case of deaths ascribed to "congenital debility," may include many which are largely the result, and fairly to be ascribed to the effect of improper feeding. For this term, according to the rules for classification in use by the Bureau of the Census, includes all deaths under one year, ascribed to such vague causes as debility, marasmus, emaciation, malassimilation, causes which—in any case if they occur after the first or second month of life—are probably often due to improper feeding or to digestive disorders.

In this connection it may be noted that the mortality from congenital debility from the second to the twelfth months among artificially fed infants was 10.7 times that among breast-fed infants, a rate entirely comparable with the excess mortality from gastric and intestinal diseases. On the other hand, in the first month the excess mortality from this cause was only 3.1 times that among breast-fed infants.

Although part of the excess mortality from "congenital debility" appearing among the artificially fed is probably due to inaccurate return of the cause of death, nevertheless, a considerable excess mortality from other causes peculiar to early infancy, including premature birth, injuries at birth, and malformations, can be explained only on the hypothesis that there is a tendency for infants who are physically weak to be artificially fed in larger numbers than are normally endowed infants. The fact that such a tendency existed would not preclude the conclusion, of course, that the chances of life of these infants were greater if they were breast fed, than if they were artificially fed.

The present tabulations permit the study of the relative prevalence of artificial feeding in three groups for which the infant mortality rates from all causes, and in particular from causes peculiar to early infancy, are exceptionally high. These groups are the twins and triplets, the prematurely born, and the infants whose mothers died within one year after confinement. The rates of mortality for these groups were respectively, 363, 541, and 450 per 1,000, as compared with an average rate for all infants of only 111. The rates from causes peculiar to early infancy for these groups were respectively 187, 405, and 257, as compared with an average rate for all infants of 36. Whether these rates may be considered to indicate innate physical weakness of the infants, or to show the consequences of the physical condition of their mothers, or the results of unfavorable environmental conditions, and to what degree each factor contributes to the group result—these questions are difficult to answer satisfactorily; for the present purpose it is sufficient to show whether the mortality among artificially fed infants is increased by the inclusion among them of a disproportionate number of infants in these groups, in which the mortality rates for other reasons than feeding are exceptionally high.

The evidence shows that in each of these groups infants were artificially fed in larger numbers than in the group as a whole. Thus, of the months lived up to the end of the ninth by twins and triplets 45.1 per cent. were months of artificial feeding, as compared with only 24.5 per cent. of the months lived by single infants. Similar percentages for premature and for infants whose mothers died were 43.8 and 82.8, as compared with 24.9 per cent. for all infants.

Though the group of artificially fed infants is thus seen to be overweighted by twins and triplets, by infants prematurely born, and by infants whose mothers died, each of these groups forms a very small proportion of the total. Thus, the twins and triplets were only

2 per cent. of the total live births, the prematurely born infants 5 per cent., and the infants whose mothers died only 0.6 per cent. Of the months of artificial feeding up to the ninth 3.3 per cent. were lived by twins and triplets, 6.5 per cent. by infants prematurely born, and 1.4 per cent. by infants whose mothers died within one year after confinement, as compared with 1.1 per cent., 2.5 per cent., 0.1 per cent., of the months of breast feeding lived respectively by twins and triplets, by infants prematurely born and by infants whose mothers died.

In the entire group of infants, as already noted, the mortality rates among the artificially fed averaged 3.9 times as high as among the breast fed. Among the plural births alone the mortality of artificially fed infants averaged 3.6 times, while among the single births alone it averaged 3.8 times as high as among the breast-fed infants. Combining the results, instead of 1047 only 279.2 deaths would have occurred in the entire group of infants artificially fed, if the rates among single and plural breast-fed infants had been applied to the single and plural artificially fed infants respectively. The excess mortality thus averaged 3.75 times as high, even after the elimination of the influence of the excess weighting of the group of artificially fed infants by twins and triplets.

The effect of eliminating the influence of the other factors is similarly relatively slight. When the influence of the overweighting of the artificially fed group by infants who were prematurely born is eliminated, the excess mortality among the artificially fed is reduced only from 3.9 to 3.56. When the influence of the overweighting of the group of artificially fed by infants whose mothers died is eliminated, the excess mortality is reduced only from 3.9 to 3.69.

Since a much larger proportion of plural births are prematurely born than of single births, the reduction in the relative excess mortality resulting from eliminating the influence of prematurity may in part duplicate the reduction resulting from eliminating the influence of plural birth. Nevertheless, when both these factors are eliminated, the reduction in the relative excess mortality is only from 3.9 to 3.4. When all three factors are eliminated the mortality among artificially fed infants still averages 3.3 times that among breast-fed infants.

This analysis, though somewhat technical, shows clearly that the effect of the overweighting of the group of artificially fed infants by infants in groups, having high mortality rates, is of comparatively minor importance, in comparison with the marked excess mortality associated directly with the kind of feeding.

Reference has already been made to the possible influence which the tendency to feed artificially infants in certain groups, characterized by high mortality rates in larger proportions or beginning at earlier times than other infants, might have in increasing the apparently cumulative effect of the type of feeding. Of the infants who were artificially fed, beginning in the first month, 12.4 per cent. were either prematurely born or were twins or triplets, as compared with an average of 7.3 per cent. of those beginning artificial feeding in the second and third months, an average of 5.0 per cent. of those beginning in the fourth to seventh months, and an average of only 3.8 per cent. of those beginning in the eighth to twelfth months.

To eliminate the effect of such an influence, Table VII exhibits the monthly death rates among artificially fed single infants who were born at term, and whose mothers lived throughout the year following their births, classified according to the month when artificial feeding commenced. The effect of eliminating the three groups, previously discussed, is to reduce materially the excess mortality among infants who were artificially fed from the first month, as compared with that among infants whose artificial feeding commenced later. This elimination, however, does not alter the general conclusion previously reached. In the fifth month, for example, the rate of infant mortality is higher the longer the period of previous artificial feeding. The tendency already noted for the rate to increase relatively to the rate among breast-fed infants, immediately following the beginning of artificial feeding at an early age, is still found to hold true for this group of infants. Among the infants whose exclusive artificial feeding commenced in the second month, for example, a mortality rate prevailed in that month which was 1.9 times the rate among breast-fed infants; the rate in the third month, however, was 6.3 times, in the fourth month 5.6 times, and in the fifth month 6.9 times the corresponding rates among breast-fed infants. Among the infants whose exclusive artificial feeding commenced in the third month, a rate prevailed in that month which was 3.9 times, in the fourth month 5.0 times, and in the fifth month 6.1 times the corresponding rates among breast-fed infants. In fact, the elimination of the group of premature and plural births and of infants, whose mothers died within one year after the births, appears not to weaken, but even to

TABLE VII.

Monthly death rates, by duration of previous artificial feeding, omitting prematurely born infants, twins and triplets, and infants whose mothers died within the year following confinement; infants in eight cities.

Month of life.	Deaths in month per 1,000 infants* surviving at beginning of month.		Deaths in month per 1,000 infants* surviving at beginning of month, who were artificially fed from—								
	Exclusively breast fed.	Exclusively artificially fed.	First month.	Second month.	Third month.	Fourth month.	Fifth month.	Sixth month.	Seventh month.	Eighth month.	Ninth and later months.
First	11.7	32.2	32.2								
Second	4.9	20.3	27.4	9.3							
Third	3.4	17.9	18.2	21.3	13.3						
Fourth	3.1	17.6	28.1	17.4	15.6	1.0					
Fifth	2.9	17.0	21.4	20.3	17.9	12.3	2.0				
Sixth	1.9	17.2	18.3	18.1	21.5	19.7	9.9	4.7			
Seventh	1.8	12.7	15.6	19.3	8.8	10.6	12.0	9.4	3.7		
Eighth	3.0	8.6	9.8	11.2	7.7	10.7	6.1	4.7	7.4	2.7	
Ninth	3.2	9.8	12.3	13.3	13.4	11.9	6.1	11.9	—	2.7	
Tenth	3.4	8.8	12.5	4.8	10.2	10.9	10.2	9.6	13.0	5.3	1.9
Eleventh	2.5	6.4	6.3	11.6	8.0	3.3	8.3	7.3	3.8	5.4	4.0
Twelfth	4.5	5.7	11.5	3.9	6.9	2.2	6.3	12.3	—	—	4.2

strengthen the evidence that the type of feeding has a cumulative effect during the early months of life.

Two other questions remain for consideration. Is the excess mortality among artificially fed infants due in any part to a tendency for infants in the low income groups, or for infants of nationalities for which the mortality rates are high, to be artificially fed in larger proportions than those in groups for which the mortality rates are low? In other words, is the excess mortality, already shown to exist among infants artificially fed, due to a difference that depends upon the type of feeding, or is it merely a reflection of differences in mortality connected with other causes, such as race or income?

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* Omitting prematurely born infants, twins and triplets, and infants whose mothers died within year following confinement.

The differences in the prevalence of artificial feeding in the several race and nationality groups are exhibited in Table VIII. The French-Canadian group had the largest proportion of artificially fed infants, and the Polish and Jewish had the lowest proportions. The nationalities with mortality rates above the average included the French-Canadian, the Polish, the Portuguese and the colored; of these two had high and two had low proportions artificially fed. Evidently the differences between the several groups tend at least in part to offset one another. When allowance is made, therefore, for differences in the prevalence of artificial feeding in the several nationality groups, the excess mortality ratio among the artificially fed as compared with the breast-fed infants is slightly, but only very slightly, increased, from 3.9 to 4.0. Hence, the correction for nationality does not appreciably affect the result.

TABLE VIII.

Prevalence of artificial feeding, by nationality of mother; infants in eight cities.

Color and nationality of mother.	Months lived up to the end of the ninth.		
	Total.	Months of artificial feeding.	
		Number.	Per cent.*
All mothers	192,212.5	47,842	24.9
White	180,397.5	45,516.5	25.2
Native	102,285.5	28,962	28.3
Foreign	78,112	16,554.5	21.2
Italian	11,943	1,563	13.1
Jewish	10,688	1,207	11.3
French-Canadian	8,666	3,813.5	44.0
German	6,524	1,405.5	21.5
Polish	10,391.5	1,155.5	11.1
Portuguese	5,410	1,725.5	31.9
Other	24,471.5	5,677.5	23.2
Not reported	18	7	—
Colored	11,815	2,325.5	19.7

* Not shown where base is less than 100.

The relative ratio between the average mortality among artificially fed and that among breast-fed infants varies considerably from group to group, as shown in Table IX. The excess mortality among artificially fed was only 2.2 times that among breast fed in the Italian group, one in which both rates were relatively low; while among the native white the ratio rose to 5.3 and among the German to 5.6.

TABLE IX.

Relative excess mortality among partly breast-fed and exclusively artificially fed, as compared with breast-fed infants, by color and nationality of mother; infants in eight cities.

Color and nationality of mother.	Deaths among infants.					
	Partly breast fed.			Exclusively artificially fed.		
	Expected.	Actual.	Ratio.	Expected.	Actual.	Ratio.
All mothers	207.7	269	1.3	261.2	1,047	4.0
White	172.4	240	1.4	234.6	963	4.1
Native	53.3	91	1.7	94.1	503	5.3
Foreign	119.1	149	1.3	140.5	460	3.3
Italian	19.8	17	.9	13.7	30	2.2
Jewish	12.8	6	.5	5.5	16	2.9
French-Canadian	10.4	19	1.8	39.4	95	2.4
German	6.4	8	1.3	6.2	35	5.6
Polish	24.4	39	1.6	12.3	60	4.9
Portuguese	10.1	24	2.4	17.0	73	4.3
Other	35.2	36	1.0	46.4	151	3.3
Colored	35.3	29	.8	26.6	84	3.2

So far as earnings of fathers are concerned, artificial feeding appears to be more prevalent in the groups with high earnings which have comparatively low infant mortality rates. Thus, of the months up to the end of the ninth lived by infants whose fathers earned under $550, 21.8 per cent. were months of artificial feeding as compared with 30.0 per cent. of the months lived by infants whose fathers earned $1250 and over.

When allowance is made for this greater tendency toward artificial feeding in the higher income groups, the relative excess mortality among artificially fed as compared with breast-fed infants is slightly increased. As stated above (p. 684) for the first nine months of life this relative excess was 5 times; but, when the data for one city for which earnings are not exactly comparable with the others are omitted, this relative excess is slightly altered to 5.1 times. Making the allowances for the negative correlation between artificial feeding and earnings the excess mortality ratio is raised from 5.1 to 5.3. The correction resulting from this correlation is thus seen to be relatively slight.

The relative excess mortality ratio of artificially fed infants in each of the principal earnings groups is shown in Table X. The ratio is much higher, 6.3, in the group with earnings "under $550" than in the group with earnings of $1,250 and over, where it was only 4.1. It may well be that this difference in the ratio corresponds to differences in the type of artificial feeding or to differences in the care with which feeding is given in the various earnings groups.

TABLE X.

Relative excess mortality during first nine months among artificially fed as compared with breast-fed infants, by earnings of father; infants in seven cities.

Earnings of father.	Deaths among artificially fed infants during first nine months.*		
	Expected.†	Actual.	Ratio.‡
Total	147.0	778	5.3
Under $550	50 4	315	6.3
$550–$849	62.3	313	5.1
$850–$1,249	26.5	118	4.5
$1,250 and over	7.8	32	4.1

SUMMARY.

In conclusion, the analysis has shown that artificial feeding, as actually practiced in typical city populations, is associated with a mortality between three and four times as high as the mortality among breast-fed infants. This excess mortality is not to be explained either by the slight overweighting of the group of artificially fed with infants in certain groups characterized by high mortality rates; and it appears in all nationality and in all earnings groups, though with variations depending probably upon the particular conditions prevailing in the groups. It would be of especial interest to follow through these variations by nationality and earnings to discover, if possible, the causes of these differences. And it would be of great importance to demonstrate statistically the influence upon mortality of the different types of artificial feeding, and whether in case of each of these types there appeared the cumulative effect that characterizes the group of artificially fed infants in the present study.

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* Excluding infants in no earnings and earnings not reported groups.

† Expected, if the rates for breast-fed infants in each earnings group had prevailed among the infants artificially fed, in the same group.

‡ Ratio of actual to expected deaths.

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